THE UNIVERSITY *of* EDINBURGH

Factors determining the progression of non-alcoholic fatty liver disease; the role of abnormal fatty acid and glucocorticoid metabolism

David P Macfarlane

Endocrinology Unit
Queen's Medical Research Institute
University of Edinburgh
PhD Thesis 2011

Acknowledgments

This work would not have been possible without the help and support of a number of individuals. I would like to start by thanking my supervisors Brian, Ruth and John. Firstly, Brian and Ruth, in the Endocrinology Unit, for seeing through my interview skills and giving me the opportunity to work with them. They have given me a tremendous amount of support and mentoring over the past few years. In particular, Ruth inducted me into the laboratory and taught me a great deal about laboratory techniques, whilst both always seem to have found time for me in their busy schedules. In addition, I would like to thank John Iredale, in the Centre for Inflammation Research, for his support in helping me obtain my Wellcome Trust Fellowship, and for his helpful advice thereafter.

I would like to thank Nik Morton in the Molecular Metabolism group without whose expertise my studies would not have been feasible. A number of other individuals in the Centre for Cardiovascular Science also deserve recognition for their help and advice including Moffat Nyirenda, Rudolph Riemersa, Shareen Forbes, Scott Denham, Jill Harrison, Dawn Livingstone, Eva Zou, Alison Ayres, Carolynn Cairns, and Rachel McDonnell, not forgetting Heather Laing.

I thank the funding bodies for supporting this work. Firstly, the British Heart Foundation, under whom I was initially funded for my first year, and secondly the Wellcome Trust who were generous enough to offer me a Clinical Training Fellowship and who also fund the core mass spectrometry laboratory.

I need to thank my mum and dad for their support, advice and encouragement over the past 33 years or so! I`m not sure what I would be doing had it not been for their helpful steering as a teenager.

I would of course like to thank my wife Louise for her love and support, and for letting me have some time off my new parenting duties to eventually complete this thesis. Finally, thank you to my amazing daughter Jessica for her unconditional love!

CONTENTS

Page

ABBREVIATIONS

11β-HSD1	11beta-hydroxysteroid dehydrogenase type 1
ALT	alanine aminotransferase
ASO	anti-sense oligonucleotide
ATGL	adipose triacylglyceride lipase
BMI	body mass index
CCl_4	carbon tetrachloride
CDD	choline deficient diet
cDNA	complementary deoxyribonucleic acid
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DNL	*de novo* lipogenesis
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
FFA	free fatty acid
GCMS	gas chromatography mass spectrometry
GH	growth hormone
GR	glucocorticoid receptor
HPA	hypothalamic-pituitary-adrenal
HPLC	high pressure liquid chromatography
HSL	hormone sensitive lipase
id	internal diameter
IL-1β	interleukin-1β
IL-6	interleukin-6
IL-8	interleukin-6
LPL	lipoprotein lipase
MMP	matrix metalloproteinases
MCDD	methionine and choline deficient diet
mRNA	messenger ribonucleic acid
NAD	nicotinamide adenine dinucleotide
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced)
NAFLD	non-alcoholic fatty liver disease

NASH	non-alcoholic steatohepatitis
NFκB	nuclear factor kappa-light-hain-enhancer of activated B cells
PC	phosphatidylcholine
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PPAR	peroxisome proliferator activated receptor
RNA	ribonucleic acid
ROS	reactive oxygen species
αSMA	alpha smooth muscle actin
SNP	single nucleotide polymorphism
TG	triglyceride
TGFβ	transforming growth factor beta
THE	tetrahydrocortisone
THF	tetrahydrocortisol
TNFα	tumour necrosis factor alpha
TIMP	tissue inhibitor of matrix metalloproteinases
TTR	tracer to tracee ratio
VLDL	very low density lipoprotein
v/v	volume/ volume
w/v	weight/ volume

FIGURES

CHAPTER 1-INTRODUCTION

CHAPTER 2-MATERIALS AND METHODS

CHAPTER 3-SUSCEPTIBILITY TO STEATOHEPATITIS AND HEPATIC FIBROSIS IN CHOLINE ± METHIONINE DEFICIENT MODELS OF FATTY LIVER IN MICE

TABLES

Publications arising from this thesis to date (August 2011)

Macfarlane DP, Forbes S, Walker BR (2008) Glucocorticoids and fatty acid metabolism in humans: fuelling fat redistribution in the metabolic syndrome. *Journal of Endocrinology* 197; 189-204.

Macfarlane DP, Zou X, Andrew R, Morton NM, Livingstone DEW, Aucott RL, Nyirenda MJ, Iredale JP, Walker BR (2010) Metabolic pathways promoting intrahepatic fatty acid accumulation in methionine and choline deficiency; implications for the pathogenesis of steatohepatitis. *American Journal of Physiology-Endocrinology and Metabolism.* 300; E402-409.

MEETING ABSTRACTS

Macfarlane DP, Nyirenda MJ, Zou X,Aucott RL, Raubenheimer PJ, Michailidou Z, Andrew R, Iredale JP, Walker BR. Distinct susceptibility to steatohepatitis and fibrosis in dietary models of non-alcoholic fatty liver disease in mice associates with alterations in intra-hepatic glucocorticoid metabolism. *Proceedings of the Endocrine Society's 93rd annual meeting, Boston, USA, 2011. Poster presentation for American Endocrine Society (ENDO) meeting, Boston, USA, June 2011*

Macfarlane DP, Morton NM, Nyirenda MJ, Andrew R, Iredale JP, Walker BR. Primary differences in intrahepatic fatty acid metabolism rather than increased peripheral flux of free fatty acids contribute to the pathogenesis of steatohepatitis in dietary models of non-alcoholic fatty liver disease but do not influence the progression to liver fibrosis. *Poster presentation at the Medical Research Society spring meeting, London, February 2010*

Macfarlane DP, Morton NM, Nyirenda MJ, Andrew R, Iredale JP, Walker BR. Use of stable isotopes to measure abnormalities of fatty acid metabolism in mouse models of fatty liver disease. *Oral presentation at the British Mass Spectrometry Society Specialist Interest Group on Stable Isotopes (BMSS-ISOSIG), Glasgow, January 2009*

Macfarlane DP, Andrew R, Morton NM, Nyirenda MJ, Iredale JP, Walker BR. Differences in hepatic fatty acid metabolism explain contrasting body weight and steatohepatitis in dietary models of non-alcoholic fatty liver disease in mice. *Endocrine Abstracts **19:** P156. Poster presentation at the Society for Endocrinology British Endocrine Society, Harrogate, March 2009*

Macfarlane DP, Nyirenda MJ, Andrew R, Walker BR. Differences in fatty acid metabolism in dietary models of fatty liver disease may underlie progression to steatohepatitis. *Oral communication at the Scottish Society for Experimental Medicine Meeting, Dundee, May 2008*

Macfarlane DP, Nyirenda MJ, Andrew R, Walker BR. Factors determining progression of non-alcoholic fatty liver disease. *Oral presentation at the Junior Diabetes Forum, Dunkeld, November 2007.*

Declaration

This thesis is the original research of the author unless otherwise stated. Analysis of plasma leptin and liver function tests in Chapters 3 and 4 were kindly undertaken by Eva (Xiantong) Zou and staff at the MRC Centre for Reproductive Biology respectively. Routine tinctorial liver stains and paraffin embedding were performed in the Queen`s Medical Research Institute Histology service. Lastly, the real time PCR and protein assays in Chapter 5 were undertaken by Carolynn Cairns.

This work has not been submitted previously at this or any other university for a higher degree.

Signature:

…….

David P Macfarlane

Abstract

Obesity and insulin resistance are associated with a constellation of features including hypertension, dyslipidaemia, type 2 diabetes, and premature cardiovascular disease, collectively termed the *metabolic syndrome*. Non-alcoholic fatty liver disease (NAFLD) represents the hepatic component of this syndrome, incorporating a spectrum of liver disease with increasing morbidity and mortality, from simple steatosis, to non-alcoholic steatohepatitis (or NASH), fibrosis, cirrhosis and ultimately hepatocellular carcinoma. However, factors influencing this progression are incompletely understood.

In this thesis I sought to investigate pathways which promote hepatic inflammation and fibrosis by studying two contrasting dietary models of NAFLD in mice in which the risk of hepatic inflammation, insulin resistance and fibrosis differ; namely the methionine and choline deficient diet (MCDD) which induces steatohepatitis, hepatic insulin resistance, and weight loss, and the choline deficient diet (CDD) which may be protected from insulin resistance, and leads to steatosis without inflammation or weight loss. I investigated the possible molecular mechanisms underlying these differences, and whether they influenced progression to hepatic fibrosis induced by carbon tetrachloride (CCl_4).

1 Chapter 1-Introduction

1.1 NON-ALCOHOLIC FATTY LIVER DISEASE

This thesis concerns the mechanisms underpinning non-alcoholic fatty liver disease (NAFLD) and its progression to non-alcohoic steatohepatitis (NASH). In this chapter, I will provide a summary of the clinical problems encompassed by NAFLD, followed by a detailed discussion of what is currently known about underlying mechanisms. Much of this has been learned from animal models, and these are reviewed, with a particular focus on the dietary models used in this thesis involving methionine and/or choline deficiency. One of the potentially modifiable mechanisms influencing liver fat accumulation and subsequent injury involves glucocorticoid signaling. The chapter therefore also includes a review of glucocorticoid biology and its influence on features of NAFLD.

1.1.1 NAFLD: Background and terminology

The association of obesity with intrahepatic triglyceride accumulation and inflammation has been known since the 1950s (Zelman 1952). However, it was not until several cases of liver failure were reported following intestinal (jejuno-ileal) bypass surgery for morbid obesity that interest in the condition began to develop (Peters et al. 1975), with the term non-alcoholic steatohepatitis or NASH being introduced by Ludwig in 1980 (Ludwig et al. 1980). The broader term non-alcoholic fatty liver disease (NAFLD or *fatty liver*) has subsequently been adopted to incorporate a wider spectrum of fatty liver disease, and is often used when histological information is unavailable. NAFLD is classically associated with features of the so-called *metabolic syndrome*, but other secondary causes of fatty liver disease, including alcohol, should be excluded (see Table 1.1) (Adams et al. 2005).

No longer considered a benign condition, NAFLD represents a spectrum of disease with the potential to progress from uncomplicated intracellular triglyceride (TG) accumulation or simple *steatosis* (with or without elevated aminotransferases), to steatohepatitis, liver fibrosis, cirrhosis and ultimately hepatocellular carcinoma, with associated increases in morbidity and mortality (Matteoni et al. 1999).

Table 1.1 Secondary causes of intrahepatic triglyceride accumulation

Cause	**Examples**
Drugs	Tamoxifen, amiodarone
Rapid weight loss	Following ileo-jejunal bypass
Pregnancy	
Cushing's syndrome	
Burns patients	
Rare syndromes	Lipodystrophies, abetalipoproteinaemia

1.1.2 NAFLD and the metabolic syndrome

Insulin resistance is characterised by reduced peripheral (predominantly muscle) glucose uptake in response to insulin, and/or increased hepatic glucose output secondary to reduced hepatic insulin sensitivity, although effects on other metabolic pathways such as fatty acid and triglyceride metabolism may also be a feature. Insulin resistance is associated with a constellation of features including obesity (especially visceral obesity), hypertension, dyslipidaemia, abnormal glucose metabolism, and low birth weight, known as the metabolic syndrome and increases the risk of cardiovascular disease (Marchesini et al. 2001). NAFLD is now widely regarded as the hepatic component of the metabolic syndrome (Marchesini et al. 2001). Hepatic triglyceride accumulation correlates with measures of peripheral (Marchesini et al. 1999), adipose and hepatic insulin resistance (Seppala-Lindroos et al. 2002). However, it is not clear whether insulin resistance is a contributing factor or a consequence of fatty liver and the mechanisms will be discussed later in this chapter.

1.1.3 Epidemiology

In parallel with the epidemic of obesity and type 2 diabetes the prevalence and awareness of NAFLD are increasing, so much so that it is now a major public health concern. NAFLD is now the commonest reason for referral to a liver clinic for investigation of deranged liver function tests, and is thought to underlie the majority of cases of cryptogenic cirrhosis resulting in end stage liver failure (Clark et al. 2003). Indeed, fatty liver may recur in these patients following transplant (Ong et al. 2001).

The true prevalence of NAFLD is difficult to determine as patients are often asymptomatic and liver function tests are often normal (Adams et al. 2005). Estimates therefore depend on a number of factors e.g. the population studied, the screening investigation, and diagnostic criteria used. In an urban population in the United States 33.6% of individuals were found to have elevated liver fat using magnetic resonance proton spectroscopy, the most sensitive screening investigation, performed in over two thousand participants in the Dallas Heart Study (Browning et al. 2004). In this study certain ethnic subgroups (Hispanics in particular) had a higher risk of NAFLD, whereas African Americans were relatively protected. Increased liver fat was also more common in males than females. Other estimates suggest up to 75% of patients with type 2 diabetes and almost 100% of patients with both type 2 diabetes and morbid obesity have fatty liver (Luyckx et al. 1998;Tolman et al. 2007).

1.1.4 Histological Classification

The liver acinus incorporates a functional zonation, with each zone differing in susceptibility to toxic and ischaemic damage, as well as fat accumulation (Brunt et al. 1999).

Zone 1: closest to the portal tracts is the first to receive toxic insult
Zone 2: intermediate region between zones 1 and 3
Zone 3: adjacent to the central venule, most prone to ischaemia (centrilobular necrosis) and fat accumulation

NAFLD is characterised histologically by macrovesicular steatosis, with intracellular lipid accumulation displacing the hepatocyte nucleus to the periphery of the cell, causing a "chicken wire" appearance. Classically, this begins in zone 3, before overflowing to the rest of the acinus, with greater than 5% steatosis by weight required to confirm the diagnosis (Adams et al. 2005). In contrast to imaging studies, only a liver biopsy can provide information on the presence of inflammation or the stage of fibrosis, which may alter patient monitoring and surveillance. Biopsy also excludes other causes of liver disease when the diagnosis is uncertain.

Additional histological features in NAFLD may include Mallory bodies (eosinophilic intracellular inclusions composed mainly of ubiquinated keratin), ballooning degeneration of hepatocytes (possibly indicating necrotic cell death), and perisinusoidal fibrosis, although these changes can regress if cirrhosis ensues (Matteoni et al. 1999). Hence the diagnosis can be obscured in those with very advanced disease and a liver biopsy is required to differentiate between simple steatosis and more advanced NAFLD. Other findings include lipogranulomas composed of chronic inflammatory cells and Kupffer cells.

Lobular inflammation, consisting of polymorponuclear leucocytes and monocytes, is usually mild and localised to the sinusoids in steatosis. NAFLD activity scoring systems have been introduced to classify the severity of NASH which is usually associated with necrosis and more widespread inflammation (Kleiner et al. 2005). Histological scoring systems also measure the extent of associated fibrosis (Kleiner et al. 2005). Perisinusoidal fibrosis usually begins in zone 3, although can progress to periportal fibrosis and bridging fibrosis may ensue. Differing patterns of fibrosis (periportal) have also been described in overweight NASH patients suggesting a different pathogenesis (Ratziu et al. 2000). A number of classification systems grading the degree of steatosis and necroinflammation, as well as the stage of fibrosis have been proposed (Brunt et al. 1999;Kleiner et al. 2005).

1.1.5 NAFLD and liver fibrosis

Liver fibrosis, and its end stage cirrhosis, can be considered a chronic healing response to repeated liver injury. Worldwide, hepatitis B and C, and alcohol predominate as causes of liver injury (Bataller and Brenner 2005), but liver fibrosis secondary to NAFLD is increasingly recognised, and causative factors may co-exist e.g. fatty liver exacerbates hepatitis C induced liver injury (Vidali et al. 2008). Fibrotic liver disease is a major burden on health care systems globally. It is estimated that 1% of the population may have cirrhosis (Schuppan and Afdhal 2008), although the prevalence of lesser degrees of fibrosis will be greater. Given the increasing prevalence of obesity and type 2 diabetes, it is likely that we will see an increasing burden of liver disease secondary to NAFLD.

Liver fibrosis is characterised by scarring, as normal liver tissue is replaced with the deposition of extracellular matrix (ECM) rich in fibrillar collagens (predominantly type 1 and type 3) (Schuppan et al. 2001). This disrupts the normal liver architecture, with loss of sinusoidal fenestrations, as the space of Disse becomes filled with scar tissue preventing sinusoidal functioning. Fibrosis may progress to classical nodular cirrhosis, with islands of hepatocytes surrounded by collagenous fibrous septae, ultimately producing liver dysfunction associated with portal hypertension, liver failure, and increased risk of hepatic carcinoma (Schuppan and Afdhal 2008).

Cirrhosis is classically described as a diffuse, irreversible process, for which no cure exists except liver transplant. However, there is increasing evidence of histological improvement if the underlying fibrogenic factor is eliminated e.g. with antiviral drugs in cases of viral hepatitis, and removal of the toxic insult in animal models (Dienstag et al. 2003;Iredale 2007). Given this potential for improvement, it is essential we increase our understanding of the pathogenesis of liver fibrosis, including in NAFLD, to develop effective and targeted treatments.

1.1.6 Natural History of NAFLD

Due to the invasive nature of liver biopsy, and the likely high proportion of the population with uncomplicated steatosis, only limited information is available on the natural history of NAFLD. Nevertheless, it is now accepted that individuals can progress from simple steatosis, to steatohepatitis, steatohepatitis with fibrosis and cirrhosis (Sanyal 2002). The progression from steatosis to steatohepatitis is thought to be rare, with one small study of 40 patients finding no progression over a 10 year period, although one patient did develop fibrosis (Teli et al. 1995). However, reports of patients with steatosis progressing to steatohepatitis do exist, especially following gastric bypass surgery (Luyckx et al. 1998), and more recent estimates suggest somewhere between 12-40% of patients with steatosis will progress to NASH with early fibrosis over an 8-13 year interval (de Alwis and Day 2008).

Progression to liver fibrosis is more likely once NASH has developed. In one prospective study of 26 patients with biopsy proven NASH, followed for up to nine years, 27% progressed to fibrosis and 19% to cirrhosis, with the remainder not demonstrating progressive histology (Younossi et al. 2002). Other series have shown that up to 50% of NASH patients develop progressive fibrosis (Hui et al. 2005), although the interpretation of these results is limited due to study design including a selected patient population, with limited follow up. Once cirrhosis has developed there is a 10 year risk of liver related mortality of 30-40% (Edmison and McCullough 2007), and a 7% risk of hepatocellular carcinoma (de Alwis and Day 2008).

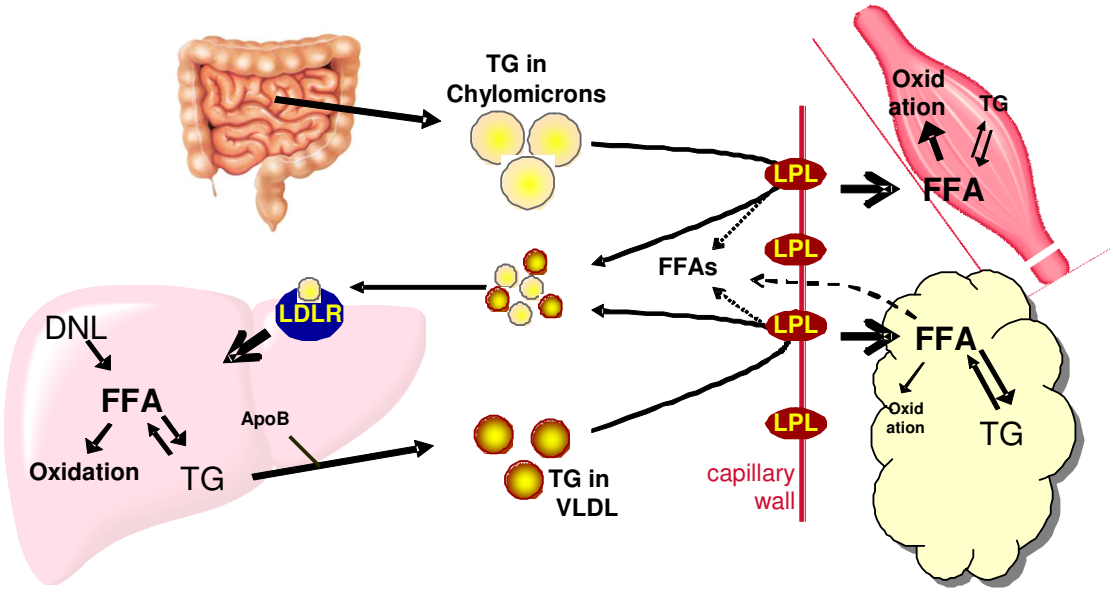
1.2 FATTY ACID AND TRIGLYCERIDE METABOLISM

Abnormalities of free (or non-esterified) fatty acid (FFA) and triglyceride (TG) metabolism are likely to influence the pathogenesis of NAFLD. I will therefore provide an overview of FFA and TG metabolism and their hormonal regulation, followed by a discussion on how alterations in their metabolism could contribute to the pathogenesis of NAFLD and insulin resistance.

Along with glucose, FFAs are a major source of fuel for oxidative metabolism (Coppack et al. 1994). FFA trafficking and metabolism is summarised in Figure 1.1. FFAs are insoluble in plasma and therefore require to be either complexed to albumin or esterified with glycerol to form TGs for packaging in lipoprotein particles before transport in the circulation (Spector 1975). The majority of fatty acids in humans are derived from dietary sources and stored as TGs in adipose tissue, or in small amounts in liver and muscle. *De novo* lipogenesis (DNL) is an additional source of FFAs (Hellerstein 1999). Circulating chylomicron and very low density lipoprotein (VLDL) TGs, delivered from the gut and liver respectively, are hydrolysed by lipoprotein lipase (LPL) located on the luminal surface of the capillary endothelium (Linder et al. 1976). This allows FFAs to be taken up into cells, either by a passive or facilitated process (Garfinkel et al. 1976;Kalant and Cianflone 2004), and then re-esterified to TGs within tissues. However, FFAs released from circulating TGs can also *spillover* into the plasma FFA pool without prior transportation into tissues (Coppack et al. 1992), and recent data suggests that direct uptake of circulating FFAs from plasma may also occur (Kratky et al. 2005).

Figure 1.1 Summary of free or non-esterifed fatty acid (FFA) metabolism and sources of intra-hepatic fatty acids and triglycerides (TG)

Overview of fatty acid and triglyceride metabolism. FFAs reach the liver from a number of sources including hydrolysis of circulating TGs, or adipose stores. Within the liver de novo lipogenesis is another source of fatty acids. TG is exported from the liver in the form of VLDL which transports TG to tissues. FFAs are an important fuel for oxidative metabolism e.g. in muscles. DNL; de novo lipogenesis, LPL; lipoprotein lipase, VLDL; very low density lipoprotein, LDLR; low density lipoprotein receptor



TGs form an efficient energy store, containing more than twice as many calories as glycogen or protein per gram, with less water content (Hillgartner et al. 1995), and are hydrolysed to release FFAs and glycerol (plus di- and mono-acylglyceride intermediates), a process known as *lipolysis*. Complete lipolysis of TGs yields three FFAs and one glycerol molecule. There are two fates for FFAs mobilised from stored TGs: one is β-oxidation within the mitochondria to generate ATP, and the second is re-esterification to TGs. As FFA release generally exceeds their oxidation, re-esterification to TGs is a key factor regulating local FFA levels, e.g. in the liver, where FFAs can be incorporated into VLDL TG or the TG storage pool (Coppack et al. 1994).

Until recently, hormone sensitive lipase (HSL) was believed to be the main enzyme responsible for adipose tissue lipolysis (Miyoshi et al. 1988). However, it is now apparent that HSL acts to hydrolyse diacylglycerides released following prior hydrolysis of the first TG ester bond by adipose triacylglyceride lipase (ATGL) (Zimmermann et al. 2004). Single nucleotide polymorphisms in the gene encoding ATGL are associated with increased risk of type 2 diabetes, providing support for a role of abnormalities of lipolysis in the pathogenesis of insulin resistance (Schoenborn et al. 2006).

1.2.1 Acute regulation of fatty acid metabolism

Given their central role in the crucial process of cellular energy provision, it is not surprising that fatty acid metabolism is tightly regulated. The processes controlling the switch between predominant lipolysis/fatty acid oxidation during fasting and predominant lipid storage/glucose oxidation following feeding are determined principally by insulin and catecholamines, as follows.

1.2.1.1 Adipose and intravascular lipolysis

Lipolysis is closely regulated in a reciprocal, tissue specific manner such that following a meal, when glucose and chylomicrons are in abundant supply, adipose tissue LPL is upregulated, whereas its activity in muscle is suppressed (Fielding and Frayn 1998). Insulin is the major hormone encouraging lipolysis of circulating TG rich lipoproteins, whilst also suppressing release of FFAs from adipose tissue (Coppack et al. 1994) and promoting re-esterification of FFAs within adipocytes (Campbell et al. 1992). In contrast, during the post-absorptive period, when insulin levels are low, circulating adrenaline and locally produced nor-adrenaline are the main stimulators of adipose tissue lipolysis (Coppack et al. 1994).

Additional paracrine, autocrine and hormonal factors may be important in regulating lipolysis but their significance *in vivo* is less clear. *In vitro* studies suggest that the regulation of LPL expression and activity is complex (reviewed in (Mead et al. 2002)), and occurs in part by post-translational modification (Ong and Kern 1989).

Furthermore, local conditions including blood flow may influence the efficiency of lipoprotein metabolism by LPL (Coppack et al. 1994), and hence the degree of spillover of FFAs into the circulation (Samra et al. 1996b).

1.2.1.2 De novo lipogenesis

Acetyl CoA derived from glycolysis, via pyruvate, can be utilised to synthesise FFAs under the regulation of insulin dependent SREBP1c and glucose responsive ChREBP transcription factors (Postic and Girard 2008). Acetyl CoA carboxylase and fatty acid synthase are two key enzymes catalysing DNL, and require an ample supply of substrates including acetyl CoA, NADPH and ATP to allow the synthesis of FFAs (Berdanier 1989). Both liver and adipose tissue are capable of synthesising fatty acids *de novo.* However, although the biosynthetic pathways of DNL are well characterised, and rodent studies suggest that a large number of regulatory dietary and hormonal factors may be important (reviewed by (Hillgartner et al. 1995)), the physiological significance of factors influencing DNL in humans is less clear. Indeed, except during embryogenesis and lactation, the importance of DNL as a source of lipids is a matter of debate (Hellerstein 1999). Broadly speaking, however, insulin promotes DNL, whereas adrenaline, glucagon and FFAs themselves inhibit DNL (Hillgartner et al. 1995).

1.2.1.3 Mitochondrial β-oxidation

Mitochondrial β-oxidation is essentially the reverse process of lipogenesis, producing acetyl CoA and ATP from the oxidation of FFAs. However, whereas lipogenesis is a cytosolic process, FFAs first require to be converted to acyl CoA intermediates for transfer into mitochondria for oxidation (McGarry and Brown 1997). Carnitine palmitoyl transferase 1 (CPT 1) undertakes this transport process, and is the initial rate limiting step in FFA oxidation. Its activity is tightly regulated such that malonyl CoA, the principle intermediate in DNL, inhibits CPT 1 (Zammit 1999). Therefore, factors influencing lipogenesis also influence mitochondrial oxidation, with insulin inhibiting and adrenaline stimulating oxidation of FFAs; these factors also influence substrate availability for oxidation (Blaak et al. 1994;Sidossis et al. 1996).

1.2.1.4 Hepatic VLDL metabolism

The liver acts as an important buffer for FFAs, temporarily storing them in the cytosolic TG pool prior to transport/cycling back to adipose tissue stores by VLDL (Gibbons et al. 2004). Hepatic VLDL assembly and secretion is a complex and incompletely understood process intimately associated with apolipoprotein B metabolism (Olofsson and Boren 2005). In simplistic terms insulin acts to inhibit VLDL assembly and secretion directly, whilst also reducing glucose and portal FFA flux which normally promote VLDL export post prandially (Gibbons et al. 2002).

1.2.2 Long-term regulation of fatty acid metabolism

The physiological control of energy metabolism requires us to achieve not only appropriate responses to acute feeding and fasting, but also appropriate longer term adaptations to the nutritional environment. Longer term regulators of fatty acid metabolism are less well understood than the acute adaptations which accompany the normal daily cycle of fasting and feeding. Growth hormone (GH), although not as potent and rapidly acting as adrenaline, may be important during exercise and fasting, increasing adipose lipolysis over a period of 2-3 hours (Moller et al. 1990). Accordingly, chronic GH deficiency is associated with accumulation of TG in adipose tissue (Rosen et al. 1993). More recently there has been interest in macrophage derived cytokines e.g. interleukin-6 (IL-6), and tumour necrosis factor α (TNFα) modulating adipose tissue lipolysis, although their physiological significance remains unclear (Jensen 2003;van Hall G. et al. 2003). Other stimulators of lipolysis include thyroid stimulating hormone, parathyroid hormone and cholecystokinin, all of which act through Gs coupled receptors. Inhibitors of lipolysis include insulin-like growth factor 1, adenosine, alpha adrenergic agonists, and prostaglandin E_2 (Coppack et al. 1994).

Members of the nuclear hormone receptor family play a key role in 'setting the scene' for metabolic control in the longer term. These include peroxisome proliferator activating receptors (PPARs) and sex steroid receptors, which may exert their effects by altering adipocyte differentiation as much as by altering fatty acid

metabolism. The potency of these effects is illustrated by the dramatic differences in fatty acid metabolism between men and women (Koutsari and Jensen 2006) and by the effects of PPARγ agonists on fat distribution (Shadid and Jensen 2003).

Glucocorticoid receptors are also expressed in adipose tissue and liver and glucocorticoid hormones may be important in both the acute and chronic regulation of fatty acid trafficking and metabolism, and in influencing adipose tissue differentiation and function. This will be discussed later in this chapter.

1.2.3 Hepatic fatty acid metabolism

Understanding FFA and TG metabolism within the liver, at both the "whole body" and molecular level may provide insights into the pathogenesis on NAFLD. Both will be reviewed here. Figure 1.1 demonstrates that the liver plays a central role in lipid metabolism. Intrahepatic FFAs reach the liver from a number of sources including the diet (via chylomicrons), from hydolysis of other circulating TGs (e.g. low density lipoprotein), from plasma FFAs derived from hydrolysis of adipose TG stores, and from *de novo* lipogenesis with the liver. In contrast, the liver can metabolise FFAs through oxidative pathways and can export FFAs incorporated into VLDL TG.

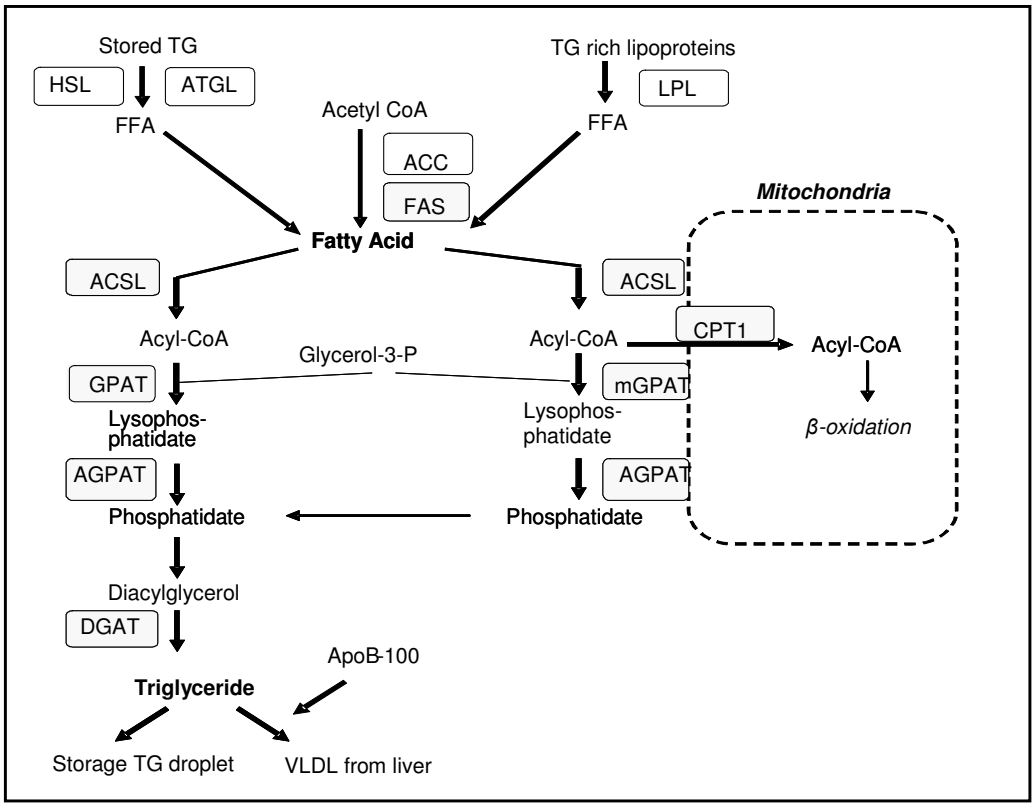
1.2.3.1 Triglyceride synthesis

The final common pathway of triglyceride (or triacylglycerol) synthesis, involving FFAs from any source, requires the incorporation of fatty acyl CoAs (activated FFAs from acyl CoA synthases) onto a glycerol-3-phosphate spine. Glycerol-3-phoshate acyltransferase (GPAT) incorporates the first fatty acid (as an acyl CoA), and diacylglycerol acyltransferase (DGAT) the final step (see Figure 1.2). It is proposed that GPAT is the rate limiting step in TG biosynthesis, although DGAT may also be important in determining the flux of FFA into TGs (Yen et al. 2008), with GPAT activity elevated in diet induced obesity, high sucrose diet, *ob*/*ob* mice and drug induced causes of fatty liver (Gudbrandsen et al. 2006;Linden et al. 2004;Postic and

Girard 2008). However, the supply of FFAs is also likely to be crucial and potentially rate limiting (Bradbury and Berk 2004).

Figure 1.2 Pathways and enzymatic regulation of triglyceride synthesis

Enzymatic pathways involved in FFA and TG metabolism. Abbreviations: ACC; acetyl-CoA carboxylase, ACSL; long chain acyl-CoA synthetase, AGPAT; 1-acylglycerol-3-phosphate O-acyltransferase , ATGL; adipose triacgylceride lipase, CPT1; carnitine palmitoyl transferase, DAG; diacylglycerol, DGAT, DAG O acyltransferase, DNL; de novo lipogenesis, FAS; Fatty acid synthase, GPAT; glycerol-3-phosphate O-acyltransferase, HSL; hormone sensitive lipase, LPL; lipoprotein lipase, mGPAT; mitochondrial glycerol phosphate acyltransferase,



At least four GPAT isoforms exist, with the mitochondrial isoform (GPAT1) being most abundant in the liver (Linden et al. 2004). Both GPAT1 and the FFA transporting enzyme carnitine palmitoyltransferase 1 (CPT1) are located on the outside of mitochondria, and overexpression of GPAT1 suppresses fatty acid oxidation by increasing incorporation of FFAs into TG (Linden et al. 2004). At least

two specific DGAT enzymes exist; DGAT1 and DGAT2 (Yen et al. 2008). DGAT2 is probably the dominant enzyme *in vivo*, as DGAT2 null mice die within a few days of birth and have virtually no fat, with low hepatic and plasma FFAs and TGs (Stone et al. 2004).

1.2.3.2 FFA oxidation

The process of β-oxidation, producing acetyl CoA and reducing equivalents (NADH, $FADH_2$) from fatty acids, takes place in the mitochondria and peroxisomes, whereas the minor pathway of microsomal ω-oxidation takes place in the endoplasmic reticulum (also known as the microsome) (Reddy and Rao 2006). In health, mitochondrial β-oxidation is the predominant pathway for metabolising short chain (<C8), medium chain (C8-C12) and long chain fatty acids (C12-C20), with the subsequently produced acetyl CoA and reducing equivalents leading to the production of ATP via the electron transport chain (Reddy and Rao 2006). Peroxisomal β-oxidation is required for the metabolism of very long chain fatty acids (>C20), and other more complex and toxic compounds such as dicarboxylic fatty acids produced by ω-oxidation, prior to mitochondrial oxidation (Reddy and Rao 2006). These alternative pathways are cytochrome P450 (CYP2E1 and CYP4A) dependent and can be upregulated in NAFLD, which may be significant as they are less efficient and generate reactive oxygen species (ROS) (Robertson et al. 2001).

Following CPT1-mediated entry into mitochondria, the first step in β-oxidation requires dehydrogenation of fatty acyl CoAs by acyl CoA dehydrogenases (Reddy and Rao 2006). This is followed by a number of reactions catalysed by mitochondrial trifunctional protein (MTP) to produce acetyl CoA for entry into the Kreb`s cycle. Peroxisomal oxidation first relies on acyl CoA oxidases prior to a number of further reactions, again catalysed by a single protein (L-bi/multifunctional enzyme [L-PBE/MFP1]), which produces shortened acyl CoAs for β-oxidation (Reddy and Rao 2006).

1.2.3.3 Triglyceride export

Pre-existing cytosolic TG stores account for the majority of FFAs incorporated into TG for VLDL export (Bradbury and Berk 2004). The underlying process is incompletely understood, but the enzyme hepatic triacylglyceride hydrolase (TGH), located on the endoplasmic reticulum, has been proposed to catalyse the mobilisation of hepatic TG stores via a lipolysis and re-esterification pathway involving acyl transferases (Gilham et al. 2003). Within the liver, TG rich VLDL particles are packaged with apolipoprotein B100 (apoB100), and other apolipoproteins (e.g. apoE, apoC-II, and apoB48 in mice expressed from the *Apobec-1* gene) for export into the circulation. This process is dependent on microsomal triglyceride transfer protein (MTTP) located within the endoplasmic reticulum, the significance of which will be discussed below (Raabe et al. 1999).

Phosphatidylcholine is another component essential for VLDL export (Li and Vance 2008). Choline, and methionine (and methyl-folate) metabolism is closely linked with phospatidylcholine synthesis and given that the dietary models of NAFLD in this thesis involve choline and methionine deficiency, their metabolism will be discussed in some detail here.

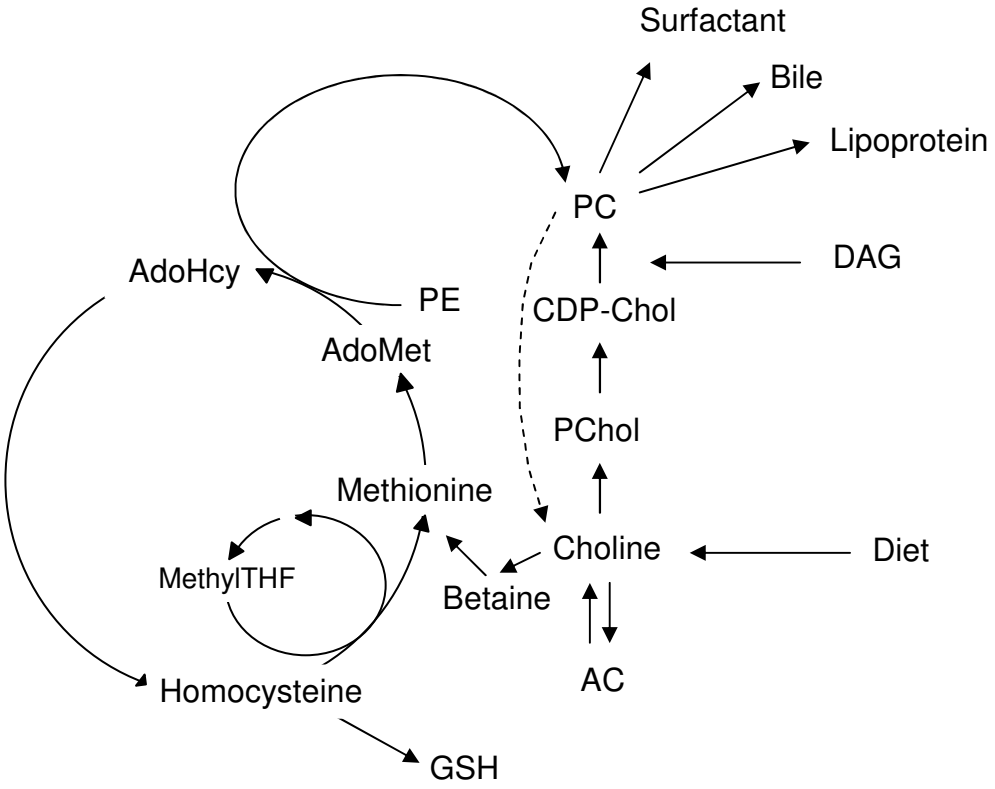
Choline is an integral component of phospatidylcholine and acetyl choline. It is therefore essential for normal membrane function, neurotransmission, and transmembrane signalling, and plays an important role in lipid transport, as phosphatidylcholine is required for hepatic VLDL export (Zeisel and Blusztajn 1994). Choline is obtained mainly from the diet, although in mammals it can also be biosynthesised via the conversion of phosphatidylethanolamine (PE) by PE N-methyltransferase (PEMT), requiring S-adenosyl methionine (from methionine) as a methyl donor. Choline can also be obtained by the action of phospholipases on pre-existing phosphocholine, as well as from catabolism of acetyl choline (Li and Vance 2008). In animals, including humans, the PEMT pathway is only relevant in the liver where it produces 30% of hepatic phosphatidylcholine, although this is upregulated in choline deficiency (Li and Vance 2008). Figure 1.3 demonstrates that the final step

in phosphatidylcholine synthesis involves the reaction of DAG with CDP-choline (by CDP-choline:1,2-diacylglycerol cholinephosphotransferase).

Choline is a quaternary amine containing three methyl groups and functions as an important methyl donor following its conversion to betaine. This allows conversion of homocysteine to methionine (by betaine: homocysteine methyltransferase). Similarly methionine is regenerated from homocysteine using methyltetrahydrofolate as a methyl donor (Figure 1.3). As early as the 1930s choline deficiency was shown to induce fatty liver in rodents (Best et al. 1932), and the deficiency of other components in methyl metabolism including methionine, folate and vitamin B12 exacerbates fatty liver, hence the collective term *lipotrope.*

Figure 1.3 Pathways of choline and methionine metabolism

Adapted from Zeisel and Blusztajn (Zeisel and Blusztajn 1994). AC, acetyl choline; DAG, Diacylglcerol; GSH, Glutathione; HC, homocysteine; PC, phosphatidyl choline; Pchol, phosphocholine; CDP-Chol, Cytidinediphosphocholine; AdoMet, S-Adenosylmethionine; AdoHcy, S-adenosylhomocysteine, PE, phosphatidylethanolamine; MethylTHF, Methytetrahydrofolate.



1.3 PATHOLOGY ASSOCIATED WITH ALTERED FATTY ACID METABOLISM: INSULIN RESISTANCE AND NAFLD

As discussed, NAFLD is considered the hepatic component of the metabolic syndrome which putatively has resistance to the metabolic actions of insulin as its central mechanism. The resistance to insulin`s actions in muscle, adipose and liver are likely to contribute to the metabolic consequences of this syndrome. However, it is not clear how many of the features of this syndrome are a cause versus a metabolic consequence of insulin resistance. Indeed, increased hepatic TG accumulation can be associated with improved insulin sensitivity suggesting that NAFLD may represent a protective mechanism against the toxic effects of intermediaries of TG metabolism. Conversely increased liver fat accumulation may lead to insulin resistance. Furthermore, abnormalities of hepatic fatty acid metabolism and/or insulin resistance may influence the development of NASH and liver fibrosis. In the following sections I will discuss the evidence that abnormalities of fatty acid metabolism can contribute to both insulin resistance and the development and progression of NAFLD.

1.3.1 Fatty acid metabolism and insulin resistance

There is a large body of research implicating FFAs in the pathogenesis of insulin resistance, either through direct mechanisms or via associated increased intramyocellular lipid accumulation (Boden 1997). In fact, elevated plasma FFAs predict the development of type 2 diabetes (Pankow et al. 2004) independently of insulin resistance and insulin secretory defects (Charles et al. 1997). Moreover, short term reductions in FFAs (using the lipolysis inhibitor acipimox) can improve insulin sensitivity in obese patients with diabetes (Santomauro et al. 1999).

Over 40 years ago Randle *et al* proposed that increased FFAs competitively inhibit the oxidation of glucose, contributing to the development of insulin resistance in rat muscle (Randle et al. 1963). However, more recent studies (Boden and Shulman 2002), corroborated by *in vivo* magnetic resonance spectroscopy findings (Petersen and Shulman 2002), suggest that inhibition of glucose metabolism by FFAs is

secondary to impaired insulin signalling rather than a direct result of substrate competition e.g. FFAs are a source of oxidative stress, which activates the proinflammatory cytokine nuclear transcription factor β (NFκB), potentially interfering with insulin signalling (Itani et al. 2002), and also reducing insulin binding with its receptor *in vitro* (Svedberg et al. 1990). Additionally, elevated FFAs can increase rates of hepatic gluconeogenesis and VLDL secretion (Chen et al. 1999;Lewis et al. 1995), although the mechanisms contributing to this are not well understood (Krebs and Roden 2005). Finally, effects on pancreatic beta cells may contribute to hyperglycaemia. Elevated FFAs are toxic to pancreatic beta cells in rodents, but in humans increasing FFA levels within the physiological range for up to 48 hours produced an increase in glucose induced insulin secretion, although clearly longer term changes may differ (Boden et al. 2001).

The source and site of pathologically important elevated FFA levels remains controversial. A popular concept is of *hyperlipolysis* in visceral adipose tissue, increasing the flux of FFAs through the portal circulation and inducing hepatic insulin resistance, but the evidence for this is not conclusive. Although visceral obesity does correlate with FFA delivery to the liver (Nielsen et al. 2004), its contribution to systemic FFA levels is small (Miles and Jensen 2005). Subcutaneous adipose tissue is arguably more likely to influence peripheral insulin resistance in upper body obesity. Although visceral fat is more lipolytically active per kilogram fat than subcutaneous adipose tissue, the quantity of the latter is greater, contributing to the majority of circulating FFAs (Basu et al. 2001). Recent studies suggest a key concept is a lack of metabolic flexibility, whereby suppression of FFA release in the fed state is impaired in insulin resistant subjects (Frayn 2002). A reduced intracellular capacity to switch between FFA and glucose oxidation in response to nutrient supply is also a feature of insulin resistance, although whether this is a cause or consequence of reduced insulin sensitivity is unclear (Kelley et al. 2002).

1.3.1.1 Intrahepatic lipid metabolism and insulin resistance

It is unclear whether increased intrahepatic TGs *per se* or other intermediaries of lipid metabolism lead to the development of insulin resistance associated with

NAFLD. Although reducing hepatic TGs is often associated with improved insulin sensitivity, one cannot infer causation. Indeed, evidence from specific models of NAFLD (discussed below) suggest that TG may be acting as a buffer of potentially toxic FFAs or other intermediaries of FFA and TG metabolism such as fatty acyl CoAs, or diacylglycerol (DAG) (see Figure 2.1).

Initial studies in *Gpat1* knockout mice, as would be predicted, showed accumulation of intrahepatic fatty acyl CoAs (Neschen et al. 2005). This was associated with protection from hepatic insulin resistance (measured by euglycaemic hyperinsulinaemic clamp) and TG accumulation induced by a high fat diet (rich in safflower oil) suggesting that increased fatty acyl CoAs alone do not contribute to the pathogenesis of hepatic insulin resistance (Neschen et al. 2005). However, these findings were not reproduced in *Gpat1* knockout mice fed a standard chow, with no change in insulin sensitivity (Hammond et al. 2002). In addition, impaired glucose tolerance with hyperinsulinaemia was found in *Gpat1* knockout mice fed a lipogenic high sucrose, high fat diet rich in coconut oil for four months (Hammond et al. 2005). Despite these conflicting results, hepatic overexpression of *GPAT1* in rats does lead to steatosis and hepatic insulin resistance associated with increased DAG and lysophosphatidic acid (LPA), supporting a role for these intermediaries in hepatic insulin resistance (Nagle et al. 2007). DAG is a potent activator of protein kinase Cε (PKCε, a serine/threonine kinase that interferes with insulin signalling). This model was associated with an increase in PKCε activity, but without any increase in NF-κB or TNFα gene expression, suggesting that hepatic inflammation is not relevant in this model of steatosis and insulin resistance.

Suppression of *Dgat2*, the predominant isoform in the liver, using a *Dgat* anti-sense oligonucleotide (ASO) in rats fed a high fat diet, reduced intrahepatic TGs and improved hepatic insulin sensitivity. This was despite elevated plasma FFAs and intrahepatic fatty acyl CoAs (Choi et al. 2007). Paradoxically *Dgat* "inhibition" was also associated with a reduction in measured DAG (and LPA) levels, possibly explained by reduced expression of various lipogenic genes (*Gpat1*, *Srebp1c*, *Acc*, *Scd1*) in response to the increase in intrahepatic fatty acyl CoAs (Choi et al. 2007). It

is well known that FFAs themselves can promote TG synthesis and act as ligands for transcription factors including PPARs (Schoonjans et al. 1996). *Dgat2* ASO treatment has also been shown to reduce hepatic TGs in high fat fed and *ob*/*ob* mice associated with improved insulin sensitivity (Yen et al. 2008).

In contrast, overexpression of *DGAT2* in the liver leads to the development of hepatic steatosis without altering plasma TGs and without insulin resistance, demonstrating a dissociation of these two features of the metabolic syndrome (Monetti et al. 2007). Another model with apparent dissociation of intrahepatic TG accumulation and insulin resistance is the *Mttp* null mouse (Minehira et al. 2008). These mice have impaired VLDL export and steatosis with normal body weights and intramuscular TGs, despite low plasma TGs. Hyperinsulinaemic euglycaemic clamp studies demonstrated normal hepatic and peripheral insulin sensitivity despite elevated intrahepatic DAG levels (Minehira et al. 2008) as would be predicted, in contrast to what was found with *Dgat2* inhibition (Choi et al. 2007). Lastly, the ApoB-38.9 mouse model of familial hypobetalipoproteinaemia (FHBL) is another model where hepatic TG content is not linked to insulin resistance (Schonfeld et al. 2008).

These studies demonstrate that insulin resistance is not a universal finding in NAFLD and, although it is not clear which intermediary of hepatic lipid metabolism is responsible, specific intermediaries of hepatic lipid metabolism may contribute to the pathogenesis of hepatic insulin resistance.

1.3.2 Fatty acid metabolism and steatosis

Given the intrahepatic accumulation of TG, simplistically, NAFLD can be considered an imbalance between the liver's ability to process the influx of FFAs and to export TG as VLDL. Abnormalities of fatty acid metabolism which could lead to the development of steatosis therefore include: increased delivery of FFAs; increased hepatic *de novo* lipogenesis; impaired FFA oxidation; and impaired TG export (see Figure 1.1). Given the liver's central role in metabolising lipids from peripheral

sources, adipose tissue FFA metabolism may also be of particular significance in the development of NAFLD. The role of each of these pathways will be discussed in more detail below, with reference to evidence mainly derived from animal studies.

1.3.2.1 Increased FFA delivery

A popular hypothesis linking obesity and fatty liver is an increased delivery of FFAs to the liver relative to oxidative capacity, promoting the esterification of excess FFAs to TGs. A simple overnight fast is sufficient to increase liver TGs in some animals (Yasuhara et al. 1991), presumably due to increased delivery of FFAs to the liver. Similarly, states of insulin deficiency e.g. in pancreatectomised dogs, increase FFAs and exacerbate hepatic steatosis (Scow et al. 1957). Likewise, insulin resistance at the level of the adipocyte is hypothesised to increase the flux of FFA to hepatocytes, in particular directly through the portal vein from metabolically active visceral adipose depots (Lewis et al. 1995).

High fat diets lead to the development of visceral obesity and fatty liver in certain strains of mice and rats, associated with increased plasma FFA concentrations (Koteish and Diehl 2001). Therefore, increased dietary fat intake may contribute to fatty liver through increased availability of FFAs, both of which can be prevented by exercise training in rats (Gauthier et al. 2003). However, other factors may be important in high fat diet induced fatty liver, including the type of fat (saturated versus polyunsaturated), insulin resistance/ hyperinsulinaemia, and leptin resistance/hyperleptinaemia. Similarly, overfeeding by gavage or gastrostomy produces obesity, associated with fatty liver, and insulin resistance, although also associated with steatohepatitis (Deng et al. 2005;Zou et al. 2006).

Adipose derived factors other than FFAs may also influence the pathogenesis of NAFLD in high fat feeding. Adipose tissue is also the source of adipokines, including adiponectin, leptin and resistin, which are proposed to modulate hepatic insulin resistance and fatty acid oxidation (Lafontan and Viguerie 2006). Additional adipose derived adipocytokines e.g. TNFα, IL6 and TGFβ are potential modulators of local adipose and hepatic lipid and carbohydrate (insulin signalling) metabolism,

whilst also being potent modulators of the immune system, of particular relevance to NASH (Choi and Diehl 2005).

As discussed, insulin resistance at the level of the adipocyte increases FFA flux to the liver due to impaired suppression of lipolysis. A more specific example of the relevance of extra-hepatic causes of steatosis comes from mice lacking the FFA uptake receptor CD36 which have increased circulating FFAs and develop hepatic steatosis associated with hepatic insulin resistance but peripheral insulin sensitivity (Coburn et al. 2000;Goudriaan et al. 2003). In addition, mice with adipose overexpression of the glucocorticoid regenerating enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), predicted to increase lipolysis secondary to elevated local glucocorticoid concentrations, have a three fold increase in portal vein FFA concentrations and develop fatty liver (Masuzaki et al. 2001). However, direct effects of increased portal glucocorticoid concentrations may also be of relevance (Paterson et al. 2002). Lastly, mice with Hsl deficiency, which are unable to efficiently hydrolyse adipose TGs to release FFAs, have low plasma FFAs and liver TG content (Voshol et al. 2003).

The physiological association of fasting elevations in FFAs and fatty liver, along with the above models suggest that increased FFA flux to the liver is likely to be an important factor in the development of fatty liver. However, it is difficult to completely dissociate the potential effects of FFAs on other aspects of metabolism and insulin resistance, such as on the accumulation of lipids within muscle, and the effects of FFAs on insulin signalling (Petersen and Shulman 2002). Nevertheless, the unopposed lipolysis and increased hepatic triglycerides in pancreatectomised animals provides evidence for the potential role of increased hepatic FFA delivery in the absence of peripheral insulin resistance (Scow et al. 1957).

1.3.2.2 Increased hepatic FFA synthesis

Hepatic *de novo* lipogenesis (DNL) is an additional source of FFAs for the synthesis of TGs. Rodents fed low fat/high carbohydrate diets develop hepatic steatosis, inducing multiple enzymes involved in *de novo* lipogenesis such as acetyl CoA

carboxylase and fatty acid synthase (Postic and Girard 2008). High carbohydrate/low fat diets also promote hyperinsulinaemia, paradoxically increase plasma TGs, and lead to the preferential oxidation of carbohydrates (Scribner et al. 2008). This latter change may in part relate to inhibition of the rate limiting enzyme Cpt1a, required for the transport of fatty acids into mitochondria for oxidation, by malonyl CoA, the principal intermediary in the *de novo* synthesis of FFAs (Zammit 1999).

Carbohydrate composition is important in the development of steatosis in these dietary models. Diets high in the disaccharide sucrose (table sugar), metabolised to glucose and fructose monosaccharides, strongly promote lipogenesis and (periportal) steatosis, whereas diets high in simple glucose do not lead to comparable changes, suggesting that fructose is the responsible factor in high sucrose diets (Koteish and Diehl 2001). Accordingly fructose metabolism bypasses the normal rate limiting mechanisms of glycolysis, producing lipogenic acetyl CoA and glycerol-3-phosphate, the backbone for triglyceride synthesis (Stanhope and Havel 2008). Interestingly, leptin has recently been shown to reverse the metabolic abnormalities induced by high sucrose diets in rats, demonstrating the complexity of the mechanisms involved in the pathogenesis of hepatic TG accumulation (Huang et al. 2007).

A number of transgenic mice overexpressing key enzymes involved in *de novo* lipogenesis have been created, along with attempts to knockout or knockdown many of the same genes. In keeping with the above dietary models, mice overexpressing either *a* and *c* isoforms of *SREBP*1 in a tissue specific manner under rat *Pepck* and murine aP2 promoters, in the liver and adipose tissue respectively develop steatosis, whereas reduced expression of *Chrebp* leads to a reduction in lipogenesis (reviewed in (Anstee and Goldin 2006)).

Stearoyl CoA desaturase (SCD1), catalyses the desaturation of fatty acids to monounsaturated fatty acids (MUFAs) e.g. palmitoleic (C16:1n-7) and oleic acids (C18:1n-9). For unclear reasons, *Scd*1-/- mice have low *Srebp1c* and *Chrebp* levels associated with suppressed lipogenesis and increased fat oxidation (Miyazaki et al. 2007), and are protected from steatosis (Miyazaki et al. 2007). Leptin has been

shown to suppress Scd-1 activity independently of its actions on Srebp1c, and may partly explain leptin's influence on liver TG accumulation (Biddinger et al. 2006). Similarly, increased lipogenesis, with increased fatty acid synthase and *Srebp1c* expression appears to be one of the mechanisms of TG accumulation in leptin deficient *ob*/*ob* mice, as well as leptin resistant *db*/*db* mice and *Fa*/*Fa* rats (Koteish and Diehl 2001).

1.3.2.3 Altered fatty acid esterification and triglyceride synthesis

A number of acyl transferases catalyse the final steps in TG synthesis (see Figure 2.1). Mice deficient in *Gpat1* are protected from steatosis, and have reduced plasma TGs/VLDL export (Hammond et al. 2002). In contrast, rodents overexpressing *GPAT1* in the liver have increased liver TGs and plasma TGs/VLDL export (Nagle et al. 2007). Similarly, overexpression of *DGAT2* in the liver leads to the development of hepatic steatosis, although without altering plasma TGs and without insulin resistance, demonstrating a dissociation of these two features of the metabolic syndrome (Monetti et al. 2007). This provides evidence that steatosis alone is not universally toxic and may actually protect against lipotoxicity, buffering toxic intermediates such as FFAs.

1.3.2.4 Altered fatty acid oxidation

Inhibition of FFA oxidation will lead to an increase in intrahepatic fatty acids and may contribute to oxidative stress within intracellular organelles. As discussed, CPT1 is essential for transport of fatty acids into mitochondria prior to oxidation and is inhibited by malonyl CoA (Zammit 1999), a cellular marker of *de novo* lipogenesis. Therefore impaired FFA oxidation may partially contribute to steatosis in models with significantly increased *de novo* lipogenesis such as high sucrose/fructose diets (Lambert et al. 2003). More specifically, given the requirement of CPT1 for carnitine, inhibiting carnitine biosynthesis can induce steatosis (Spaniol et al. 2001), as does CPT1 inhibition by etomoxir (Koteish and Diehl 2001). Examples of drugs impairing fatty acid oxidation include tetracycline, doxycycline, amiodarone, and tamoxifen, as well as glucocorticoids (Fromenty and Pessayre 1995;Letteron et al. 1997).

The first step in carnitine biosynthesis requires two essential nutrients; the methyl donor methionine and lysine to create trimethyl-lysine (Borum 2009). Given choline and methionine`s roles as methyl donors, discussed in detail below, deficiency of these nutrients can reduce tissue carnitine concentrations (Carter and Frenkel 1978) and has been shown to reduce CPT1 activity (Aarsaether et al. 1988). This may be important in methionine and/or choline deficient dietary models of NAFLD as discussed below. Similarly, juvenile visceral steatosis (JVS) mice have reduced activity of the (renal tubular) carnitine transporter, Octn2, and develop steatosis exacerbated by carnitine deficiency (Knapp et al. 2008)

Mitochondrial β-oxidation is reliant on a large number of enzymes many of which have been disrupted in mice and are under the regulation of PPARα transcription factor (Schoonjans et al. 1996). *Pparα* knockout mice demonstrate the importance of fatty acid oxidation in preventing steatosis, as although they do not develop fatty liver under normal fed conditions, the increased release of FFAs associated with (24hour) fasting leads to severe steatosis (Kersten et al. 1999). Male *Pparα* knockout mice are extremely sensitive to the CPT1 inhibitor etoxomir, the effects of which can be ameliorated with β-oestradiol therapy suggesting an important role for oestrogens in the regulation of fatty acid oxidation (Djouadi et al. 1998). Indeed, aromatase deficient female mice develop centrilobular steatosis which can be prevented by β-oestradiol and PPARα agonist therapy (Toda et al. 2001). This mechanism may also be partially responsible for tamoxifen associated steatosis (Fromenty and Pessayre 1995).

The oxidative pathways described above could be disrupted at a number of stages. The importance of MTP is demonstrated in mice with homozygous mutation in the *Mtp* gene which die shortly following birth (Ibdah et al. 2005). Mice heterozygous for a defect in *Mtp* develop steatosis after ~10 months, associated with hepatic oxidative stress, and insulin resistance assessed by GTT (Ibdah et al. 2005). In contrast, defects in peroxisomal oxidation are reproduced in mice lacking acyl CoA oxidase. These accumulate very long chain fatty acids in the blood, and develop steatosis which progresses to steatohepatitis (by four months of age). This then

resolves by six to seven months, possibly due to an increase in β-oxidation and hepatocellular regeneration (Fan et al. 1996;Reddy and Rao 2006).

1.3.2.5 Impaired hepatic TG export

If TG synthesis exceeds the capacity for TG export, then steatosis will develop. Processes which could contribute to impaired hepatic TG export include: decreased mobilisation of hepatic TG stores; impaired VLDL assembly; and decreased VLDL secretion (Bradbury and Berk 2004).

Certain drugs including amiodarone and tetracycline have been shown to influence MTTP activity both *in vitro* and *in vivo* in mice (Letteron et al. 2003). This is in addition to the effects of these medications on mitochondrial β-oxidation which may increase their propensity to cause steatosis. Indeed, clinical studies of an MTTP inhibitor in familial hypercholesterolaemia showed an increase in transaminases and liver fat (Cuchel et al. 2007). Carbon tetrachloride (CCl_4), a toxin commonly used to induce liver fibrosis in rodents, has also been reported to inhibit hepatic TG export, enhancing hepatic steatosis, which may be due to increased post translational MTTP breakdown (Pan et al. 2007).

Hydrolysis of stored TGs is an essential step in VLDL export. Inhibition of hepatic triglyceride hydrolase (TGH) inhibits TG and apoB export *in vitro* (Gilham et al. 2003), whereas human *TGH* transgenic mice have increased VLDL export, although hepatic TG content was not assessed (Wei et al. 2007). To date no *in vivo* studies of TGH inhibitors or molecular downregulation have been published. The role of TG hydrolysis has not been well studied in relation to NAFLD. Recently, hepatic overexpression of Hsl and Atgl, the predominant lipolytic enzymes in adipose tissue, using adenovirus transduction was shown to ameliorate steatosis in *ob*/*ob* mice (Reid et al. 2008). No increase in VLDL export was measured, but fatty acid oxidation was increased, suggesting that the action of these lipases in the liver may direct FFAs towards oxidation rather than VLDL export (Reid et al. 2008).

Both apoB100 and MTTP are essential for normal hepatic TG export. It is thought that apoB is synthesised in excess of its requirements, with post transcriptional regulation involving MTTP associated lipidation, preventing alteration of apoB100's conformation that would otherwise lead to its degradation (Tietge et al. 1999). However, ApoB deficiency does exist in humans as familial hypobetalipoproteinaemia (FHBL) and can lead to the development of steatosis (Schonfeld et al. 2003). Similarly, the ApoB-38.9 mouse model of FHBL develops steatosis due to impaired VLDL export, and perhaps as an adaptive mechanism, has suppressed *de novo* lipogenesis and esterification, with unchanged fatty acid oxidation (Lin et al. 2002). In humans, deficiency of MTTP is responsible for the syndrome of abetalipoproteinaemia, an autosomal recessive condition characterised by very low apoB containing lipoprotein levels, fat and fat soluble vitamin malabsorption, and fatty liver (Wetterau et al. 1992). Liver specific inactivation of the *Mttp* gene in mice lowers plasma apoB100 levels by over 95%, associated with markedly reduced plasma lipids, and causes the development of hepatic steatosis (Raabe et al. 1999). In contrast, overexpression of the *MTTP* gene leads to an increase in VLDL export and plasma TGs suggesting that Mttp may be rate limiting (Tietge et al. 1999).

ApoE also appears to play an important role in VLDL export (Mensenkamp et al. 2001). ApoE deficient mice develop perivenous steatosis when fed normal chow, "cured" by reintroduction of hepatic apoE (Mensenkamp et al. 1999), whereas overexpression increases VLDL export (Tsukamoto et al. 2000). The mechanism is unclear, but appears to affect quite a late event in VLDL assembly in the endoplasmic reticulum, independent of Mttp activity (Mensenkamp et al. 2001).

Excluding the specific models discussed in this Section (1.3.2.5), the significance of impaired VLDL export in fatty liver is unclear. Hepatic VLDL export is increased in humans with NAFLD and insulin resistance (Fabbrini et al. 2008). MTTP activity may actually increase in an attempt to increase TG export, perhaps in response to increasing TG accumulation (Bartels et al. 2002;Carpentier et al. 2002). However, decreased - or perhaps failure to upregulate - VLDL export may exacerbate fatty

liver e.g. in *ob*/*ob* mice (Li et al. 1997). Polymorphisms in the *MTTP* gene have been demonstrated in human NASH (Namikawa et al. 2004), in which there is also evidence of impaired apoB export (Charlton et al. 2002).

Altered VLDL export has also been implicated in the pathogenesis of methionine and/or choline deficient models of fatty liver, although other mechanisms may also be important in these two contrasting models.

1.3.2.6 Methionine and/or choline deficiency

Choline and methionine are key micronutrients required for phosphatidyl choline synthesis and are essential for VLDL synthesis (Li and Vance 2008). Deficiency of these components leads to fatty liver in rodents, possibly due in part to effects on TG export. It has been widely quoted in the literature that TG accumulation in CDD and (extrapolating from this) MCDD is the result of impaired VLDL export (reviewed in (Kulinski et al. 2004)). This primarily comes from the work of Lombardi *et al* (Lombardi et al. 1968) employing the use of a radioactive palmitate tracer in CDD fed rats. The reduced incorporation of tracer into plasma TGs compared to control rats, with a greater delay in reduction of radioactivity from the liver, has been interpreted as impaired VLDL export (Lombardi et al. 1968). However, this could also simply imply an increased contribution of plasma FFAs to the accumulating hepatic TG pool in the CDD rats, with less radiolabelled VLDL TG exported per gram of stored hepatic TG. More recently *in vitro* studies, in hepatocytes isolated from CDD fed mice, have demonstrated normal VLDL export with choline deficiency alone (Kulinski et al. 2004).

Differences between rodents fed a choline deficient diet (CDD) and a methionine and choline deficient diet (MCDD) are summarised in Table 1.2**.** A choline deficient diet (CDD) rapidly (<1 week) leads to the development of fatty liver in rodents, initially periportally (zone 1), before it rapidly spreads into other areas. In rats, prolonged administration (>12 weeks) leads to the development of liver cirrhosis, and spontaneous hepatic carcinogenesis occurs after 30 weeks of choline deficiency

(Nakae 1999). As a result of this latter finding, CDD has traditionally been used as a model of liver carcinogenesis rather than fatty liver.

Table 1.2 Comparison on choline ± methionine deficient models of fatty liver in rodents

	CDD	**MCDD**
Initial TG accumulation	Zone 1	Zone 3
Spontaneous fibrosis	>12 weeks in rats	Yes
Impaired VLDL export	Unclear	Yes
Hepatic insulin resistance	No	Yes
Steatohepatitis	No	Yes
Weight loss	No	Yes

Figure 1.3 demonstrates that in the absence of dietary choline animals can still generate choline via the methionine dependent PEMT pathway. As a result of this animals are able to upregulate this pathway to maintain phosphatidylcholine synthesis required for VLDL export (Kulinski et al. 2004). In addition, upregulation of the CDP-choline pathway, increasing phosphatidylcholine synthesis, may divert potentially toxic intermediaries such as FFA and diacylglycerols which may explain the improved insulin sensitivity seen in mice placed on a high fat diet and fed a CDD (Raubenheimer et al. 2006).

The cause of steatosis in this model is unclear. Gene expression studies have suggested that *de novo* lipogenesis and fatty acid oxidation are unaltered by choline deficiency, whereas the process of re-esterification of fatty acids to TG stores is upregulated (Raubenheimer et al. 2006). One of the earliest changes noted in rats fed a CDD is subcellular damage due to the development of reactive oxygen species (Nakae 1999), and mitochondrial dysfunction secondary to free radicals has recently been shown in choline deficiency (Petrosillo et al. 2007). Hence, despite a lack of detectable changes in gene expression, altered fatty acid oxidation may be relevant in isolated choline deficiency.

The combination of choline and methionine deficiency produces a different phenotype from simple CDD. In particular, rodents fed the MCDD diet develop steatohepatitis and early fibrosis, associated with hepatic insulin resistance (Leclercq et al. 2007;Schattenberg et al. 2005). The MCDD diet also induces rapid weight loss leading to improved whole body insulin sensitivity in mice (Rinella and Green 2004), although there is also evidence from a study of pair fed rats that glucose tolerance is impaired following MCDD (Tahan et al. 2004). In contrast to isolated choline deficiency, MCDD fed rodents initially accumulate TG centrilobularly (zone 3). In this respect, histologically the MCDD model is similar to human NAFLD, but given its other phenotypic features it is perhaps a model of lipodystrophy rather the "primary" human NAFLD (Rizki et al. 2006).

MCDD fed rodents develop a profound impairment of VLDL export, associated with a fall in plasma TG concentrations (Vetelainen et al. 2007). In keeping with the loss of adipose stores, plasma FFAs are also elevated, potentially stimulating hepatic TG synthesis (Rahman et al. 2007). *De novo* lipogenesis may also be increased (Rizki et al. 2006) and there may be abnormalities of fatty acid oxidation, which given the development of steatohepatitis in this model suggests that abnormalities of fatty acid metabolism may be influential in the development of hepatic inflammation.

1.3.3 Fatty acid metabolism and non-alcoholic steatohepatitis (NASH)

It is clear from the above that multiple abnormalities of fatty acid metabolism can lead to the accumulation of intrahepatic triglyceride or steatosis. Animal models of steatohepatitis are, however, much less abundant than those with isolated steatosis but provide important insights into the mechanisms influencing progression from simple steatosis to the development of liver inflammation.

Although probably an oversimplication, the *two hit* hypothesis has been proposed to explain the progression from simple steatosis to steatohepatitis, with cytokines, FFAs and reactive oxygen species proposed as potential second hits in predisposed steatotic livers (Day and James 1998). Here I will discuss the major models of

steatohepatitis focussing on the evidence that abnormalities of fatty acid metabolism may be important in influencing the inflammatory process.

1.3.3.1 The two hit hypothesis

Ob/*ob* mice have a naturally occurring mutation (Lep^{ob}) in the gene encoding leptin, producing a truncated inactive form of the adipose derived satiety hormone, resulting in hyperphagia, obesity, and insulin resistance (Anstee and Goldin 2006;Ingalls et al. 1996). Similar phenotypes are observed in hyperleptinaemic, leptin resistant Zucker rats, with a mutation ($Lepr^{fa}$) in the gene encoding the leptin receptor (commonly referred to as Ob-R), and in *db*/*db* mice, with a homozygous mutation ($Lepr^{db}$) in the gene which encodes Ob-R. Multiple splice variants of the Ob-R exist and in *db*/*db* mice the long form important in central appetite regulation is truncated, whilst the short form is retained intact in the liver (Hoggard et al. 1997;Lee et al. 1996). *Ob*/*ob* mice have a number of abnormalities of fatty acid metabolism potentially contributing to steatosis including increased circulating FFAs, promoted by increased adipose TNFα, increased *de novo* lipogenesis, and possibly reduced fatty acid oxidation, associated with an increase in microsomal CYP2E1 and CYP4A mediated ω-oxidation (reviewed in (Anstee and Goldin 2006)).

Although older male *ob*/*ob* mice may develop mild hepatic inflammation, younger *ob*/*ob* and *db*/*db* mice do not display features of steatohepatitis (Koteish and Diehl 2001). Their steatotic livers are however particularly sensitive to toxic insults and develop steatohepatitis following lipopolysaccharide administration, ischaemia reperfusion injury and ethanol (Koteish and Diehl 2001). These findings provided the initial impetus for the "second hit" hypothesis that steatosis sensitises the liver to factors such as oxidative stress (Day and James 1998), although other factors may also be relevant e.g. leptin's modulation of the immune response. Many immune cells possess leptin receptors and leptin deficiency has immunosuppressive effects on both the innate and acquired immune response (Matarese 2000). Leptin deficiency promotes a T helper 1 (pro-inflammatory) immune response, suppressing the T helper 2 (anti-inflammatory) response (Matarese 2000), suggesting that in these models at least the two hit hypothesis is an oversimplification. Nevertheless other

models of steatosis require a second hit to induce steatohepatitis e.g. impaired VLDL export in mice with reduced expression of hepatic *Mttp* is associated with increased susceptibility to lipopolysaccharide administration (Bjorkegren et al. 2002).

1.3.3.2 Overnutrition and high fat feeding induced steatohepatitis

A diet high in saturated fat is sufficient to act as a second hit in leptin resistant *fa/fa* rats, that readily develop steatohepatitis, when placed on such a diet (Carmiel-Haggai et al. 2005). Steatosis and fibrotic changes develop in the periportal region initially in this model. Fat aussie (*foz/foz*) mice, another model of hyperphagic obesity with insulin resistance (with a deletion in the mouse *Alms1* gene), also progress from simple steatosis to fibrosing steatohepatitis when placed on a high fat diet (Arsov et al. 2006). Interestingly, this was associated with a profound reduction in adiponectin compared to chow fed controls, a factor reduced in patients with NASH, and known to upregulate *Pparα* and *Ampk* (Larter and Farrell 2006). Accordingly, *foz/foz* mice were unable to upregulate genes involved with fatty acid uptake, fatty acid oxidation, and export, suggesting an inability to adapt to a high fat diet. Despite the development of steatohepatitis there was no upregulation of microsomal oxidation and no increase in lipid peroxidation in this model (Arsov et al. 2006).

These models suggest that the progression to steatohepatitis may relate to the liver's (and adipose tissue's) ability to cope with the metabolic challenge of a high fat diet. Specifically *foz/foz* mice appeared to be unable to adapt to the increased flux of intrahepatic fatty acids by increased fatty acid oxidation and TG export.

Other investigators have attempted to induce fatty liver and steatohepatitis using high fat feeding in rodents. Lieber et al (Lieber et al. 2004) fed male Sprague-Dawley rats a synthetic liquid high fat diet (71% of calories), rich in unsaturated fat, *ad libitum* for 3 weeks which produced steatohepatitis with early fibrosis. This was associated with CYP2E1 induction, oxidative stress, increased hepatic TNFα concentrations, structural mitochondrial abnormalities, and hyperinsulinaemia (Lieber et al. 2004). Further models of high fat diet induced steatohepatitis in rats have since been published including the use of a high fat (58%), high calorie, solid diet, containing

predominantly saturated fat, more akin to the human intake (Svegliati-Baroni et al. 2006). This well characterised model initially developed a perivenular and periportal steatosis by 4 weeks followed by progression to diffuse steatohepatitis by 3 months, and zone 3 fibrosis by 6 months, and was associated with obesity and hepatic insulin resistance (Svegliati-Baroni et al. 2006).

Profound obesity can be difficult to induce in rodents when fed *ad libitum* (Zou et al. 2006), hence intragastric feeding has been employed in an attempt to simulate human obesity. Gavage of a high fat (77%) emulsion diet, high in unsaturated fat, for 6 weeks reliably induces steatohepatitis in rats (Zou et al. 2006). Similarly overfeeding C57Bl/6 mice a high fat (37%) liquid diet continuously via gastrostomy for 9 weeks has also been performed (Deng et al. 2005). This produced steatohepatitis similar to human NASH, associated with features of the metabolic syndrome, although it developed in less than 50% of mice. CYP2E1 was not induced, although CYP4A was elevated, and the development of steatohepatitis was not dependent on TNFα signalling. Steatohepatitis and fibrosis, with features of the metabolic syndrome, has also been induced in C57Bl/6 mice without gastrostomy but required 50 weeks of high fat feeding making this model of limited practical use (Ito et al. 2007).

1.3.3.3 The methionine-choline deficient diet

The methionine and choline deficient diet (MCDD) is a commonly employed model as, compared with some of the models above, it more rapidly and reliably leads to steatohepatitis. Histologically, livers in MCDD resemble human NASH, although weight loss and an associated improvement in peripheral insulin sensitivity means that it does not fully replicate the human syndrome. Therefore in an attempt to better model human disease some investigators use *db*/*db* mice, which when fed an MCDD diet do not lose weight and remain hyperleptinaemic and insulin resistant (Rinella et al. 2008).

As discussed in Section 1.3.2.6 there is evidence that abnormalities of fatty acid metabolism may be key in the development of NASH in the MCDD mode, including impaired VLDL export. In addition, MCDD is commonly described as a model of

impaired mitochondrial β-oxidation (Koteish and Diehl 2002), although the evidence for this is limited (Rinella et al. 2008;Rizki et al. 2006). It does however, appear to be a model of increased (CYP2E1 and CYP4A mediated) microsomal oxidation, leading to increased free radical generation, oxidative stress and lipid peroxidation important in the pathogenesis of inflammation and liver cell necrosis (Weltman et al. 1996). Lipid peroxidation also produces mitochondrial dysfunction, although alternatively the increased microsomal oxidation could be in response to impaired mitochondrial oxidation (Robertson et al. 2001). The TG accumulation and steatohepatitis is certainly worsened by impaired mitochondrial oxidation in PPARα null mice in the MCDD model (Kashireddy and Rao 2004), and PPARα agonists can ameliorate MCDD induced steatohepatitis, despite increased microsomal oxidation (Ip et al. 2003).

These findings support an important role for intrahepatic fatty acids in promoting steatohepatitis, possibly through their effects to increase oxidative stress. Indeed, one recent study found a 2-3 fold increase in hepatic FFA levels in MCDD fed mice (Larter et al. 2008a). This may be important as FFAs can upregulate Fas/CD95 receptors involved in apoptosis (Feldstein et al. 2003) and are directly cytotoxic via Jnk mediated mechanisms (Malhi et al. 2006). In keeping with this, previous studies have shown Jnk-1 activation to be important in the MCDD model (Schattenberg et al. 2006). *Scd-1* is downregulated in MCDD fed mice (Rizki et al. 2006) which may impair esterification of fatty acids, and is another explaination for elevated intrahepatic FFAs following MCDD.

Studies of LPL activation in the MCDD model provide further evidence for a deleterious effect of FFAs on hepatic inflammation. Activation of LPL enhanced fatty acid oxidation, upregulating PPARα, CYP4a10 and acyl CoA oxidase, and also reducing pro-inflammatory cytokine expression and hepatic inflammation (Yu et al. 2007). No differences were found in fatty acid uptake or *de novo* lipogenesis gene expression.

1.3.3.4 Other models involving methyl donor metabolism

The above discussion of the MCDD model suggests that abnormalities of fatty acid metabolism are likely to be important in its pathogenesis, but methionine also has an important metabolic role as a methyl donor (Li and Vance 2008). Other rodent models involving methyl donor metabolism have provided further insights into the MCDD model. It can be seen from Figure 1.3 that S-adenosyl methionine acts as an important methyl donor following its conversion from methionine. Mice lacking the hepatic enzyme responsible for this reaction (methionine adenosyltransferase-1A [*Mat1a*]) have markedly reduced S-adenosyl methionine in the liver and have corresponding hypermethionaemia (Lu et al. 2001). These mice develop steatosis but do not develop periportal inflammation until 8 months of age, on a normal diet. This is associated with depletion of hepatic antioxidants (glutathione) and an increase in expression of multiple genes involved with the inflammatory response including; metallothioneins 1 and 2, Fas antigen, and IL-10 (Lu et al. 2001). They also have reduced expression of CYP4As, involved in fatty acid oxidation. Analogous to the MCDD model, *Mat1a* knockout mice are sensitive to choline deficiency and develop severe macrovesicular steatosis when placed on a CDD. However, the presence of steatohepatitis was not reported, albeit after only 6 days of dietary intervention (Lu et al. 2001), which may be too early to compare with the steatohepatitis seen in MCDD (Vetelainen et al. 2007).

Pemt -/- mice lack the enzyme necessary for the methionine-dependent conversion of PE to PC and, when fed a diet devoid of choline, rapidly develop steatohepatitis. Although this is not a practical model of NAFLD, as mice die of liver failure within a few days of commencing the diet, it emphasises the importance of the PEMT pathway in maintining phosphatidylcholine synthesis when dietary choline is low (Walkey et al. 1998). These mice have similar histology and low circulating TGs to the MCDD fed mice consistent with impaired VLDL export due to reduced phosphatidylcholine availability. Antioxidant levels were not measured (although they would be predicted to be low), whereas bile acids were significantly increased, presumably relating to the role of phospholipids in the secretion of bile. This may be significant given the toxic effect of bile acids on hepatocytes (McNeilly et al. 2010).

The authors suggest altered membrane permeability may contribute to the pathogenesis of the *Pemt*-/- phenotype which may also be relevant in the MCDD model (Walkey et al. 1998).

1.3.3.5 Oxidative stress and NASH

It follows from the above the oxidative stress is likely to play an important role in the development of NASH. Indeed, patients with NASH have greater hepatic oxidative stress than those with steatosis (Sanyal et al. 2001). Glutathione is an essential hepatic antioxidant whose metabolism is closely linked with methionine as described above. Its synthesis is regulated by both glutathione synthase, and the catalytic subunit of the glutamate cysteine ligase (Chen et al. 2007). Accordingly, hepatocyte specifc disruption of the glutamate cysteine ligase encoding *Gclc* gene, leads to steatosis with progressive inflammation, lipid peroxidation, mitochondrial dysfunction and death from liver failure by one month of age in mice (Chen et al. 2007). In contrast, vitamin E supplementation reduced MCDD induced inflammation and oxidative stress, although glutathione repletion via L-2-oxothiazolidine-4-carboxylate failed to have the same effect (Phung et al. 2009).

Nuclear respiratory factor (NRF) 1 and 2 are ubiquitous transcription factors important in mitochondrial DNA transcription and replication, and the response to oxidative stress e.g. inducing glutathione synthase via the antioxidative response element (Lu 2008). Liver specific knockout of *Nrf1* in mice leads to steatohepatitis by 4 weeks of age, and the development of pericentral and pericellular fibrosis after 4 months, followed by subsequent development of hepatic adenomas and carcinomas (Xu et al. 2005). Similar to mechanisms described in MCDD mice, this is associated with CYP4A upregulation and oxidative stress, suggesting that impaired mitochondrial oxidation increases the less efficient ROS producing microsomal oxidation which, when coupled with impaired antioxidant defences, produces steatohepatitis (Xu et al. 2005). Similarly, *Nrf2* knockouts are more susceptible to MCDD induced steatohepatitis (Chowdhry et al. 2010;Sugimoto et al. 2010).

1.3.3.6 Pro-inflammatory cytokines and NASH

The pro-inflammatory cytokine TNFα is secreted from both adipose tissue and hepatocytes and is an obvious candidate to influence the development of NASH. TNFα has a number of actions including activating the protein kinase I-κ-B kinase-β (IKKβ) which interferes with insulin signalling and helps activate the nuclear transcription factor NFκB, which itself promotes expression of the pro-inflammatory cytokines IL-6 and IL-8 (Perkins 2007). Other effects include antagonism of adiponectin and mitochondrial damage, triggering apoptosis (Jou et al. 2008). Cytokines may also modulate fatty acid metabolism e.g. TNFα inhibits the effects of insulin to suppress lipolysis, and IL-6 also promotes lipolysis (van Hall G. et al. 2003), whereas conversely FFAs can also activate TNFα and NFκB (Feldstein et al. 2004).

TNFα expression is upregulated in MCDD fed mice (Yu et al. 2006), and its role in propagating steatohepatitis has been investigated using TNFα null mice, as well as TNF receptor 1 (TNFR1) knockouts. Initial findings suggested that TNFα is not essential for the development of steatohepatitis in MCDD mice, suggesting that NFκB activation is more important (Dela et al. 2005;Kirsch et al. 2006). However, a study in *Tnfr1* and *Tnfr2* double knockouts did show reduced development of steatohepatitis and liver fibrosis in the MCDD model over an 8 week period, although TG accumulation was also reduced (Tomita et al. 2006). Alanine aminotransferase (ALT) and *Tgfb* expression was unchanged between groups. Further support for a role of TNFα comes from studies using pentoxifylline which reduces TNFα production and attenuated steatohepatitis in MCDD fed mice, although glutathione levels were also increased by this treatment (Koppe et al. 2004). A role for IL-6 is supported by reduced MCDD induced liver inflammation in IL-6 null mice (Mas et al. 2009). Osteopontin is another pro-inflammatory cytokine secreted from hepatocytes and macrophages which appears to be important in the pathogenesis of NASH (Sahai et al. 2004a;Sahai et al. 2004b).

A number of other factors including angiotensin II, plasminogen activator inhibitor-1, macrophage colony stimulating factor have also been implicated in the pathogenesis of NASH (Jou et al. 2008).

1.3.3.7 Adipocytokines and NASH

The role of leptin deficiency and leptin resistance in the pathogenesis of steatosis and progression to steatohepatitis has been discussed in Section 1.3.3.1. In contrast to leptin, serum adiponectin levels inversely correlate with hepatic fat stores and insulin resistance in humans (Bugianesi et al. 2005). It is implicated in both the development and progression of NAFLD due to effects modulating lipid metabolism, promoting FFA oxidation and inhibiting lipogenesis (Yamauchi et al. 2002), and levels have been found to be lower in individuals with NASH versus steatosis (Hui et al. 2004). Adiponectin also has anti-inflammatory properties, inhibiting TNFα (Masaki et al. 2004). Indeed, recombinant adiponectin decreased steatosis and ALT in *ob*/*ob* mice (Xu et al. 2003). MCDD fed to mice unable to upregulate adiponectin leads to increased TG accumulation, steatohepatitis and fibrosis (Ikejima et al. 2007;Okumura et al. 2006). Whether these effects are due to anti-inflammatory or anti-lipogenic effects of adiponectin are unclear.

Using adiponectin knockouts and mice lacking AMP Kinase (AMPK) activity, a recent study tried to dissect the role of adiponectin in PPARγ agonist mediated improvements in MCDD induced steatohepatitis (Da Silva Morais et al. 2009). The results suggest that upregulation of adiponectin is an important mediator of the reduction in steatohepatitis seen with pioglitazone, but that suppression of SREBP1c rather than any effect on AMPK or PPARα is important (Da Silva Morais et al. 2009). Others have described reduced hepatic AMPK phosphorylation and PPARα activity in MCDD fed mice, despite elevated adiponectin levels, suggesting possible adiponectin resistance (Larter et al. 2008b;Xu et al. 2003).

1.3.3.8 Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) null mice

A final murine model of NASH which may involve abnormal fatty acid metabolism involves the *Pten* tumour suppressor gene. *Pten* encodes a lipid phosphatase involved in the regulation of pathways down-stream of phosphoinositide-3 (PI3) kinase, and helps to suppress apoptosis, cell proliferation and oncogenesis (Stiles et al. 2004). *Pten* knockout mice are unviable, whereas liver specific *Pten* knockout mice develop hepatomegaly and steatohepatitis histologically resembling human NASH by 10 weeks of age (Stiles et al. 2004). There is no preceding stage of simple steatosis however, and fibrosis does not develop until around 7 months of age. Mice are also prone to the subsequent development of hepatic adenomas and carcinomas. Of note, presumably relating to the role of PI3 kinase in insulin signalling (Stiles et al. 2004), these mice have increased hepatic insulin sensitivity, which could partially explain the increased *de novo* lipogenesis, although TG export is also increased which would normally be suppressed by insulin. Gene expression studies also suggest upregulation of mitochondrial and peroxisomal fatty acid oxidation, as well as PPARγ, associated with induction of adipogenic genes such as aP2 (Horie et al. 2004).

These mice provide evidence that insulin resistance (and hyperleptinaemia) is not essential for the development of steatohepatitis, and that retained sensitivity to insulin within the liver may be important (Stiles et al. 2004). Intrahepatic FFAs were also measured in this model although they were not elevated (Horie et al. 2004). It is not entirely clear why these mice develop inflammation/steatohepatitis and whether it is separate from the process of tumorigenesis. Potential factors include oxidative stress, and hydrogen peroxide levels are markedly increased by week 10 (7 fold greater than controls), possibly due to peroxisomal oxidation. Additionally, altered PI3 kinase signalling may activate NFκB dependent cytokine induction (Watanabe et al. 2007). Hepatic *Pten* deficiency also leads to abnormalities of cell survival signalling, but perhaps surprisingly, there is no increase in apoptosis at 10 or 40 weeks (Horie et al. 2004). DNA microarray technology has been used to assess candidate genes for inflammation in this model (Sato et al. 2006). Interestingly a number of candidates genes for inflammation included genes involved with both

fatty acid and anti-oxidant metabolism e.g. *Cpt1a*, acyl CoA oxidase, and glutathione S-transferase mu6. Other candidate genes included secreted phosphoprotein 1, pyruvate dehydrogenase kinase 4, suppressors of cytokine signalling 3, and tissue transglutaminase (Sato et al. 2006).

Interestingly, genome wide association studies have suggestd another phospholipase may be important in the progression to NASH. A SNP in the patatin-like phospholipase 3 gene (*PNPLA3*) was associated with the development of steatosis and NASH independently of BMI or insulin resistance (Romeo et al. 2008). The precise physiological role of PNPLA3 has yet to be fully elucidated but *in vitro* studies suggest it posseses both lipolytic and lipogenic properties, providing further support for a role of abnormalitites of fatty acid metabolism in the progression of NAFLD (Romeo et al. 2010).

1.3.4 Liver fibrosis in NAFLD

Over the past two decades there has been a marked increase in our understanding of the molecular mechanisms influencing hepatic fibrosis, aided by a number of available models. Models of liver fibrosis can be classified into three groups (Iredale 2007): cell culture models, studies of human biopsy/samples from hepatic resection, and animal models. Each model has their advantages and disadvantages e.g. cell culture allows the very detailed study of cell behaviour amenable to manipulation, but may be difficult to extrapolate to the complex *in vivo* environment; conversely, human studies are necessary to confirm *in vitro* or animal studies, but sequential biopsy studies are not feasible because of ethical concerns, which often limits studies to being cross-sectional in nature; animal studies can overcome some of these limitations, allowing serial profiling of tissues from homogenous groups (including genetically modified mice), but are limited due to their inherent “non-human” nature (Iredale 2007).

The two commonest models of experimental liver fibrosis are toxin induced damage (e.g. carbon tetrachloride [CCl_4]) and bile duct ligation. Other models include

expression of hepatotropic viral proteins e.g. HbSAg, with some dietary models e.g. MCDD also inducing mild fibrosis.

Current evidence suggests that inflammation drives the development of liver fibrosis, with a correlation between inflammatory features and fibrosis in human studies. Indeed, histological studies show sites of inflammation and fibrosis to be co-localised, with animal studies manipulating the inflammatory response often attenuating the fibrotic response (Iredale 2007). Both innate and adaptive immune mechanisms are involved in the response to liver injury, and involve activation of resident cells within the liver as well as inflammatory cells from extra-hepatic sources. The major cellular and molecular mediators of hepatic fibrogenesis will be discussed in brief here.

1.3.4.1 Hepatic stellate cells

Models of fibrosis have elucidated some of the key players in the development and resolution of liver fibrosis, including hepatic stellate cells. Von Kupffer first described these star shaped cells or *sternzellen* in 1876, prior to being later termed Ito cells, and currently hepatic stellate cells (HSCs) (Iredale 2007). HSCs are lipid and vitamin A containing cells residing in the space of Disse where the ECM is rich in basement membrane type collagens (Geerts 2001). In response to inflammation, or injury, they develop a myofibroblast like phenotype, expressing contractile proteins such as alpha smooth muscle actin (αSMA) (Rockey et al. 1992). Proposed activators of HSCs include platelet-derived growth factor (PDGF), endothelin-1 (ET-1), fibroblast growth factor, vascular endothelial growth factor (VEGF), and TGFβ (Eng and Friedman 2000). Conversely, activated HSCs express surface molecules, e.g. intercellular adhesion molecule-1 (ICAM-1) which can recruit immune cells.

As with all myofibroblasts, activated HSCs are essential for wound healing, proliferating and secreting fibrillar collagens in response to injury, thereby laying down the fibrotic ECM. Recent evidence suggests other sources of myofibroblasts such as the bone marrow may be important (Forbes et al. 2004). Indeed, within the liver different populations of activated HSCs may exist. HSCs also secrete factors

regulating ECM degradation (matrix metalloproteinases and their inhibitors-see below), HSC chemotaxis, and leucocyte chemoattraction (Iredale 2007). In contrast, HSC apoptosis is a feature of resolving fibrosis (Iredale et al. 1998). Activation of HSCs has been shown to predict progression to fibrosis in humans with NAFLD (Feldstein et al. 2005).

1.3.4.2 Platelets

Platelets are often recruited early in response to injury, forming part of the innate immune response and limiting blood loss. They secrete TGFβ and PDGF, both potent fibrogenic cytokines which activate HSCs (Pinzani et al. 1989).

1.3.4.3 Neutrophils

Neutrophils also represent an early component of the innate immune response and form part of the inflammatory cell infiltrate in NASH (Kleiner et al. 2005). However, studies of mice with dysfunctional or depleted neutrophils suggest that this rapid influx of neutrophils does not significantly influence the development of fibrosis (Saito et al. 2003;Xu et al. 2004).

1.3.4.4 Macrophages

Macrophages play a number of roles in hepatic fibrosis (and resolution) including, HSC activation, cell killing, regulation of inflammatory cells, and modification of the fibrotic and healing processes, and are recruited both from resident Kupffer cells as well as from circulating bone marrow derived monocytes (Henderson and Iredale 2007). Recent evidence suggests that different populations of macrophages may exist with opposing roles within the liver (Henderson and Iredale 2007). They have been broadly classified as classically and alternatively activated macrophages. The classical response leads to pro-inflammatory cytokine production in response to a number of triggers including a *Th1* CD4+ response (via interferon gamma) and degraded matrix components. In contrast, the alternatively activated macrophages depend on a *Th2* (IL-4) response, and possibly glucocorticoids, producing an anti-inflammatory response, including production of IL-10 and TGFβ (Henderson and

Iredale 2007). In the presence of myofibroblasts, this latter response is also pro-fibrotic, whereas the classical response is more lytic.

The timing of macrophage activation is also crucial. In an elegant study using transgenic mice (CD11b DTR) in which macrophages can be depleted by the administration of diphtheria toxin, Duffield *et al* (Duffield et al. 2005) demonstrated that macrophages play opposing roles at differing stages of liver injury and repair in a CCl_4 model. Macrophage depletion in the fibrotic stage reduced scarring and myofibroblast activation, whereas depletion during the recovery phase impaired resolution of fibrosis. It is unclear whether this represents differing populations or highly plastic macrophages.

Macrophages also regulate HSC apoptosis, an essential component in the resolution of fibrosis, firstly by promoting degradation of type 1 collagen which acts as a survival signal for HSCs (Friedman 2005), and also by expressing specific apoptotic signals (Issa et al. 2003). Scar associated macrophages are also the source of MMP-13, the major collagenase in mice, without which resolution of fibrosis is impaired (Fallowfield et al. 2007).

1.3.4.5 Matrix metalloproteinases

Matrix metalloproteinases (MMPs), and their inhibitors (tissue inhibitors of metalloproteinases or TIMPs, types 1-4) regulate degradation of ECM. They both play an important role in the development of fibrosis, as well as in maintaining normal tissue homeostasis (Han 2006). Indeed, polymorphisms in the genes encoding MMPs (1, 3, and 9) may influence the development of liver fibrosis in humans (Okamoto et al. 2005).

The MMPs are a family of zinc-dependent metallo-endoproteinases which can be classified as in Table 1.3 (Hemmann et al. 2007). Some overlap may occur e.g. MMP-2 also degrades fibrillar type I and II collagens, and species specific differences also exist e.g. there is no rodent homologue of MMP-1 (collagenase 1) (Hemmann et al. 2007). They are generally secreted into the extracellular space as

pro-enzymes, providing a further level of regulation, with activation by complex interactions with MT-MMPs and TIMPs. Once activated MMPs are mainly regulated by the binding of inhibitory TIMPs.

Table 1.3 Classification of matrix metalloproteinases (MMPs)

Adopted from (Consolo et al. 2009).

Class	**MMP number.**	**Alternative name**
Collagenases	MMP-1	Collagenase-1
	MMP-8	Neutrophil collagenase
	MMP-13	Collagenase 3
	MMP-18	Collagenase 4
Gelatinases	MMP-2	Gelatinase-A
	MMP-9	Gelatinase-B
Stromelysins	MMP-3	Stromelysin-1
	MMP-10	Stromelysin-2
	MMP-11	Stromelysin-3
Matrilysins	MMP-7	Matrilysin PU MP
	MMP-26	Matrilysin 2
Membrane type	MMP-14	MT1-MMP
(MT-MMPs)	MMP-15	MT2-MMP
	MMP-16	MT3-MMP
	MMP-17	MT4-MMP
	MMP-24	MT5_-MMP
	MMP-25	MT6-MMP
Others	MMP-12	Macrophage metalloelastase
	MMP-19	RASI 1
	MMP-20	Enamelysin

Degradation of the ECM appears to help initiate hepatic fibrosis, releasing hepatic stellate cells from the "normal" matrix, rich in collagen type IV, and leading to their activation (Arthur 2000). Models of fibrosis demonstrate that early changes include upregulation of MMP-13 and MMP-3 (Watanabe et al. 2000). In the later stages of fibrosis this is followed by increased expression of MMPs -2, 9 and 14 (Arthur 2000). Activated Kupffer cells are thought to be the main source of MMP-13 and MMP-9, whereas activated HSCs are the principal source of MMP-2 and MMP-3 (Arthur 2000).

Whilst some of these changes in MMP activity may lead to further HSC activation, the accumulation of ECM in fibrotic livers suggests MMP matrix degradation is inadequate. TIMP-1 and TIMP-2 are potent inhibitors of MMPs secreted by activated HSCs and in liver injury are upregulated in response to inflammatory cytokines including IL-1b, IL-6, and IL-11, as well as TNFα and TGFβ (Roeb et al. 1993). Animal studies demonstrate both TIMP-1 and TIMP-2 are upregulated rapidly in models of fibrosis, persisting for the duration of liver injury. Low levels are seen during resolution (Arthur 2000). Similarly, TIMP expression is increased in human fibrosis (Benyon et al. 1996). The ratio of MMPs and TIMPs is critical for the development and resolution of fibrosis, although factors determining their temporal expression remain incompletely understood.

1.3.4.6 Factors influencing development of fibrosis in NAFLD

The development of fibrosis in NAFLD is highly variable, with a recent study suggesting the only predictors being the presence of portal tract fibrosis on initial biopsy, or weight gain (Ekstedt et al. 2006), the latter suggesting abnormalities of fatty acid metabolism may be important. Similarly, cross sectional studies have confirmed the association between obesity and the likelihood of hepatic fibrosis, along with increasing age and diabetes (Angulo et al. 1999).

The factors determining progression to fibrosis in NAFLD are incompletely understood, but it follows that if the factors promoting (chronic) inflammation persist, associated with the appropriate millieu of cytokines, then this will also promote

fibrosis. Therefore in addition to influencing TG accumulation, abnormalities of hepatic TG metabolism may also contribute to the development of liver fibrosis. Probably the best evidence that abnormalities of intrahepatic fatty acid/TG metabolism can influence liver outcomes independently of hepatic TG levels comes from studies using *Dgat2* ASO treatment which reduced steatosis but increased fibrosis and intrahepatic FFAs in methione-choline deficient *db*/*db* mice (Yamaguchi et al. 2007). Impaired VLDL export may also sensitise the liver to toxic insults as evidenced by an increase in lipopolysaccharide induced inflammation in mice with reduced hepatic *Mttp* expression compared to controls, albeit that baseline TGs were markedly higher in the reduced *Mttp* expression cohort (Bjorkegren et al. 2002).

In keeping with the mechanisms discussed, TGFβ appears to be involved in the progession to fibrosis in MCDD induced NASH, with a TGFβ inhibitor reducing fibrosis and hepatic stellate cell activation (Uno et al. 2008). Interestingly, the inhibitor also appeared to upregulate hepatic FFA oxidation which could explain the reduced inflammation that was also seen. Accordingly, *Tgfb* expression is upregulated in MCDD rodents along with changes in both MMP and TIMP expression, with increased mRNA transcripts of *Timp-1*, *Mmp-2* and *9*, and reduced *Mmp-13* expression after 12 weeks of MCDD in rats (Mu et al. 2010). Similarly, circulating TIMP1 levels have been found to be increased in patients with NASH and fibrosis (Miele et al. 2009). The role of MMPs in NASH has not been well studied. One study found a different MMP profile in patients with NASH than in other chronic liver diseases, with increased MPP 9 and 10 expression although patients may have been at different stages in disease progression (Ljumovic et al. 2004).

Comparisons of *ob*/*ob* and *db*/*db* mice placed on an MCDD suggest that leptin is another factor important for the progression of NAFLD as *ob*/*ob* mice develop less inflammation and fibrosis than *db*/*db* and wild type mice (Sahai et al. 2004b). The authors suggest this is due to increased leptin mediated osteopontin expression via the short form leptin receptor (Ob-Ra). A number of other studies have investigated the role of leptin in the progression of liver disease which suggest that leptin is essential for the development of hepatic fibrosis, increasing TGFβ in Kupffer and

sinusoidal epithelial cells and preventing apoptosis of HSCs (Ikejima et al. 2007;Leclercq et al. 2002).

In contrast to leptin, adiponectin- which also modulates fatty acid metabolism- is an anti-fibrogenic adipocytokine, and acts to disrupt leptin signalling (Handy et al. 2010). Adiponectin, inhibits hepatic stellate cells (Adachi et al. 2008), and mice deficient in adiponectin develop worse carbon tetrachloride induced liver fibrosis (Kamada et al. 2003). In addition, MCDD fed mice unable to upregulate adiponectin also develop increased TG accumulation, steatohepatitis and fibrosis (Ikejima et al. 2007;Okumura et al. 2006).

The effects of angiotensin II on hypertension, cardiac and renal disease are well known (Timmermans et al. 1993), but the renin angiotensin system may also be important in liver disease as hepatic stellate cells possess angiotensin II type 1 receptors (Yoneda et al. 2009). Furthermore, angiotensinogen, the precursor to angiotensin II can be synthesised in adipose tissue, and may also influence fat distribution (Giacchetti et al. 2000). There is preliminary evidence that angiotensin receptor blockers may reduce hepatic fibrosis in NAFLD (Georgescu 2008).

1.4 GLUCOCORTICOIDS IN NAFLD

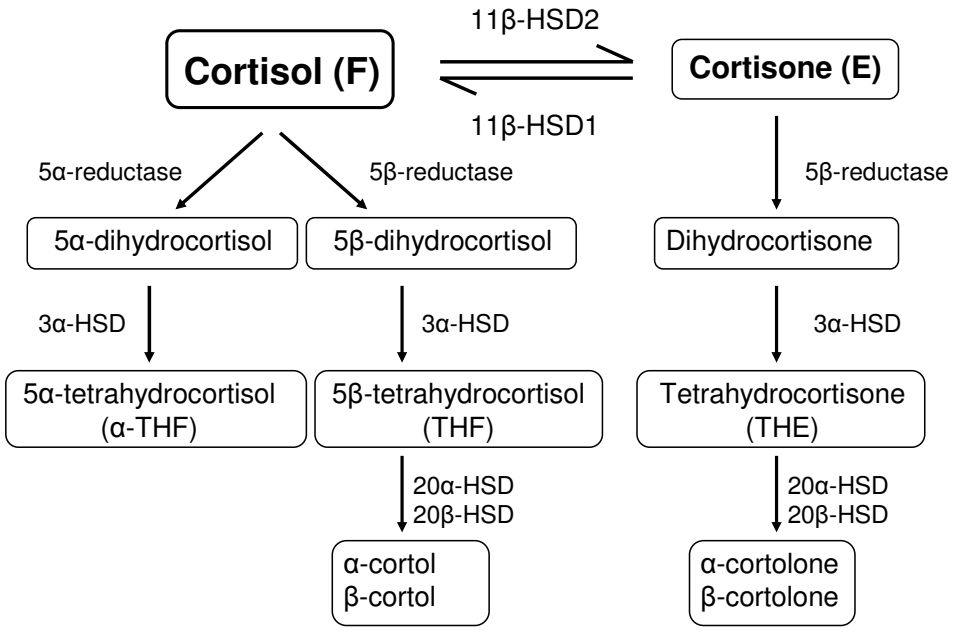
From the above it is clear that a complex series of factors may influence liver fat accumulation and the chances of progression to NASH and thence to fibrosis. Although relevant factors are numerous, a few may influence each of these processes, including fatty acid metabolism itself and hepatic inflammation. In addition to exploring the fundamental changes in fatty acid metabolism which occur in NAFLD, within this thesis I also investigated the modulatory role of glucocorticoids. Here I provide a brief review of the determinants of glucocorticoid actions in the liver and evidence that their disruption has a bearing on NAFLD.

The liver has a central role in maintaining glucose and lipid homeostasis. The liver is also the principal site for the metabolism of glucocorticoids, as shown in Figure 1.4,

and may therefore influence both local and systemic glucocorticoid concentrations. Given the wide ranging actions of glucocorticoids, abnormalities of intrahepatic glucocorticoid metabolism may lead to adverse metabolic, as well as anti-inflammatory effects.

Figure 1.4 Pathways of cortisol metabolism

Cortisol (F) and cortisone (E) can be interconverted by the 11β-hydroxysteroid dehydrogenases, and are principally metabolised by the A ring reductases (5α–reductases and/or 5β-reductase) in the liver as shown. The resultant dihydrometabolites are then metabolised to tetrahydrometabolites and excreted in the urine, mainly as conjugates.



1.4.1 Enzymes regulating glucocorticoid levels

Tissue concentrations of cortisol, the principal glucocorticoid in humans, are controlled not only by circulating levels, under the influence of the hypothalamic pituitary adrenal (HPA) axis, but also by local metabolism (see Figure 1.4). There is accruing evidence that abnormalities of glucocorticoid metabolism may contribute to the metabolic consequences of obesity. In particular, much research has focussed on

the role of the enzyme 11beta-hydroxysteroid dehydrogenase type 1 (11β-HSD1), which is highly expressed in both adipose tissue and the liver, acting to amplify local glucocorticoid levels and glucocorticoid receptor activation within tissues (reviewed in (Seckl and Walker 2001;Tomlinson et al. 2004)). Cortisol (corticosterone in rodents) is metabolised by both A-ring reductases (5α– and 5β-reductase) and 3α-hydroxysteroid dehydrogenase (3α-HSD) which convert glucocorticoids to their tetrahydrometabolites for subsequent conjugation and excretion in urine (Tomlinson et al. 2004). In contrast, cortisone (11dehydro-corticosterone in rodents) is not metabolised by 5α–reductase. (see Figure 1.4). Further metabolism by 20α– and 20β– hydroxysteroid dehydrogenases leads to the production of cortols and cortolones. Specific alterations in these enzymes may also influence circulating glucocorticoid levels and HPA axis function. It follows that alterations in both local and systemic glucocorticoid levels could influence the development and progression of NAFLD e.g. through glucocorticoid's effects on fatty acid metabolism at the adipocyte or liver, or through effects on peripheral insulin sensitivity to drive hyperinsulinaemia.

1.4.2 Glucocorticoids and fatty acid metabolism

In addition to their role in cellular growth and development, glucocorticoids have multi-systemic effects that are essential for survival in times of stress, influencing the regulation of blood pressure, salt and water balance, immune function and cellular energy metabolism. In the short-term, these acute effects contribute to adaptive responses. For example, glucocorticoids are *catabolic*, increasing availability of substrates for mitochondrial oxidation (from glucose, amino acids, and fatty acids). The consequences of failure of these adaptive responses is clearly demonstrated in the syndrome of adrenal insufficiency (in Addison's disease or ACTH deficiency), characterised by lethargy, weight loss and postural hypotension, in which severe stress or sepsis can be life-threatening (Addison 1855). If sustained in the long term, however, the effects of glucocorticoids may become maladaptive. Chronic glucocorticoid excess (Cushing's syndrome) leads to morbidity and mortality through a variety of factors including obesity, osteoporosis, hypertension,

hyperglycaemia, and impaired response to infection (Cushing 1912). These chronic adverse effects are important, not least because large numbers of patients receive treatment with synthetic glucocorticoids for a wide range of inflammatory conditions such as rheumatoid arthritis and asthma (Wei et al. 2004).

Although many of the manifestations of Cushing's syndrome are predictable on the basis of the acute effects of glucocorticoids (eg to raise blood pressure, induce insulin resistance, promote skeletal muscle wasting, and elevate plasma glucose) it appears to be a paradox that the acute lipolytic effect of glucocorticoids is not manifest in long-term weight loss in Cushing's syndrome. Indeed, chronic glucocorticoid excess in rodents usually induces weight loss rather than weight gain (Elliott et al. 1971), but some limited studies in humans with Cushing's syndrome suggest that lipolytic rates are reduced or unaltered, rather than enhanced (Birkenhager et al. 1976;Saunders et al. 1980). Moreover, in Cushing's syndrome there is marked redistribution of body fat, with accumulation in depots in the abdomen, nape of the neck and cheeks, but wasting of fat in many subcutaneous adipose compartments. There may also be excessive accumulation of triglycerides in 'ectopic' sites such as the liver in Cushing's syndrome (Rockall et al. 2003). This pattern of central fat accumulation, associated with glucose intolerance and hypertension, results in remarkable similarities between the clinical features of Cushing's syndrome and those of the Metabolic Syndrome (Reaven and Hoffman 1987), and could in part relate to glucocorticoid effects on fatty acid metabolism.

Based on the key studies summarised in Table 1.4, it is clear that glucocorticoids have important acute and long-term effects on fatty acid metabolism, but the data available at present is not conclusive. In the following sections I will review the role of glucocorticoids in modulating fatty acid metabolism.

Table 1.4 Effects of glucocorticoids on fatty acid metabolism

*Effects may be adipose tissue depot specific, ** chronic effects may relate to counter-regulatory hormone changes

	In vitro	*In vivo* in humans		11β-HSD1 inhibition
		Acute	Chronic	
Lipolysis				
- Adipose	↑ (Baxter and Forsham 1972;Slavin et al. 1994;Villena et al. 2004)	↑ (Divertie *et al.* 1991)	↑ (Gravholt et al. 2002;Krsek et al. 2006), ↔ (Miyoshi et al. 1988;Saunders et al. 1980), ↓ (Birkenhager *et al.* 1976)*	↓ (Berthiaume *et al.* 2007a)
- Intravascular	↑ (Appel and Fried 1992;Fried et al. 1993;Rebuffe-Scrive et al. 1988)	↑ (Samra *et al.* 1996a)	? ↑ **	↔ in adipose ↑ in muscle (Berthiaume *et al.* 2007a)
De novo lipogenesis	↑ (Hillgartner et al. 1995;Wang et al. 2004;Williams and Berdanier 1982)	Unknown	Unknown	↓ in adipose ↔ in liver (Berthiaume *et al.* 2007a)
Oxidation	?↑(Short *et al.* 2004), ↓(Letteron *et al.* 1997)	↑ (Djurhuus *et al.* 2002)	↔ (Burt et al. 2006;Burt et al. 2007)	↑ (Berthiaume *et al.* 2007a)
VLDL export	↑ (Wang *et al.* 1995)	Unknown	↑ (Taskinen *et al.* 1983)	↓ (Berthiaume *et al.* 2007b)

1.4.3 Acute effects of glucocorticoids on adipose tissue and intravascular lipolysis: results of *in vitro* studies

A great deal of effort has been expended on studies in cells in culture which may or may not reflect measurements undertaken *in vivo* (Lillioja et al. 1986). Re-esterification is also difficult to interpret *in vitro* due to factors such as the concentration of albumin in the culture medium (Edens et al. 1990). Most importantly, adipocyte differentiation *in vitro* is dependent on high concentrations of insulin and glucocorticoids, so that many of the effects ascribed to direct 'regulation' of gene transcription may in fact be indirect manifestations of the change in cellular phenotype in the presence of glucocorticoids. Nevertheless, *in vitro* studies have generally shown increased FFA release from adipocytes in response to glucocorticoids (reviewed in (Baxter and Forsham 1972)). Cortisol has also been shown to amplify the induction of lipolysis by catecholamines in cells primed with GH, but in the presence of insulin, reduced the basal lipolytic rate and responsiveness to catecholamines (Ottosson et al. 2000).

Both *Hsl* (Slavin et al. 1994) and *Atgl* are induced by glucocorticoids (Villena et al. 2004). In human adipocytes LPL activity and mRNA expression are also increased by glucocorticoids with differences between sexes and adipose depots studied (Fried et al. 1993;Ong et al. 1992). Omental adipose tissue has a higher concentration of glucocorticoid receptors (Rebuffe-Scrive et al. 1990) and showed a greater response to dexamethasone than subcutaneous fat, especially in men (Fried et al. 1993). Synergistic increases in LPL activity in response to insulin and dexamethasone were seen in both depots and were not fully explained by increased mRNA transcription, suggesting post-translational modification of LPL in the presence of glucocorticoids, e.g. reducing LPL degradation (Appel and Fried 1992). Conversely, the glucocorticoid antagonist RU38486 reduced adipose LPL activity *in vitro* (Ottosson et al. 1995).

These findings suggest that glucocorticoids may increase both the uptake and turnover of fatty acids in adipose tissue and, analogous to their effects to increase glucose/glycogen turnover, may modulate the dynamic responsiveness to other

stimuli such as insulin or catecholamines, potentially promoting the flux of FFA to the liver.

1.4.3.1 Acute effects of glucocorticoids on adipose tissue and intravascular lipolysis: results of *in vivo* studies

A variety of techniques are commonly employed to study lipid metabolism *in vivo*. Fatty acid turnover can be measured using isotope dilution techniques involving infusion of stable or radioactive isotopomers of fatty acids (e.g. $^{13}C_1$-palmitate) and whole body lipolysis can also be estimated using glycerol tracers to measure rates of appearance (R_a)(Wolfe and Peters 1987). Regional rates of lipolysis can be assessed by arteriovenous (A-V) sampling or microdialysis techniques measuring glycerol concentrations (Frayn et al. 1997). To minimise the influence of other hormones on lipolysis a *pancreatic clamp* can be employed by infusing somatostatin, with controlled insulin, growth hormone and occasionally glucagon replacement.

Results of short-term infusions of glucocorticoids suggest that lipolysis is increased, as in the *in vitro* experiments, but that there are counter-regulatory effects mediated by other hormones. Thus, using a pancreatic clamp with very low dose insulin replacement, the Mayo Clinic group demonstrated a 60% increase in the R_a of palmitate in response to hydrocortisone infusion after 4-5 hours, clearly demonstrating the potential of glucocorticoids to promote adipose tissue lipolysis (Divertie et al. 1991). In contrast, without a pancreatic clamp Dineen *et al* (Dinneen et al. 1993) mimicked physiological cortisol concentrations using a combination of metyrapone (to reduce glucocorticoid production) and variable hydrocortisone infusion for 15 hours, and showed no difference in the fasting R_a of palmitate during relative hypercortisolaemia, although only a single premeal measurements was taken, so subtle changes could have been missed. However, hypercortisolaemia did increase the R_a of palmitate after a mixed meal, despite a higher post-prandial insulin response suggesting resistance to inhibition of lipolysis by insulin (Dinneen et al. 1993). It is not possible to delineate whether the observed increase in the R_a of palmitate was secondary to adipose tissue lipases or increased FFA spillover from LPL acting on circulating TG, but it appears that cortisol does increase FFA release

from circulating TGs (Samra et al. 1998), suggesting a combination of increased LPL activity and increased adipose lipolysis during acute hypercortisolaemia.

Studies without tracer infusion have demonstrated that adrenaline and cortisol have synergistic effects on FFA concentrations during somatostatin administration (Pernet et al. 1986), but have no effect on the anti-lipolytic effect of insulin during hyperinsulinaemia (20mU/m^2/min) (Clerc et al. 1986). Both growth hormone (GH) and glucocorticoids may influence sensitivity to adrenaline by reducing anti-lipolytic α_2-adrenoreceptor availability (Djurhuus et al. 2004;Yip and Goodman 1999), but acute *in vivo* studies have demonstrated additive independent effects of GH and cortisol on lipolysis during a pancreatic clamp, suggesting separate mechanisms of action (Djurhuus et al. 2004). Perhaps crucially, glucocorticoids also increase pro-lipolytic β-adrenergic receptor numbers via GR dependent mechanisms (Lacasa et al. 1988;Nakada et al. 1987).

Several investigators have explored differences in glucocorticoid effects in different regions of body fat, with somewhat inconsistent results. Samra *et al* (Samra et al. 1998) induced supraphysiological plasma cortisol concentrations of ~1500nmol/l and measured increased systemic rates of lipolysis. However, subcutaneous adipose tissue lipolysis (measured by A-V difference in the anterior abdominal wall) was reduced and LPL activity (calculated from adipose tissue blood flow and TG extraction) was not altered (Samra et al. 1998), suggesting that the subcutaneous adipose tissue may be excluded from the acute lipolytic effect of cortisol. However, Djurhuus *et al* (Djurhuus et al. 2002), who also measured increased systemic rates of lipolysis, found contradictory results using microdialysis techniques: subcutaneous adipose lipolysis was increased in both abdominal and leg adipose compartments in response to hypercortisolaemia (~900nmol/) during a pancreatic clamp with low dose insulin replacement (Djurhuus et al. 2002). The discrepancies between these studies are most likely explained by the higher insulin levels in the study by Samra *et al* (Samra et al. 1998). The key experiment, comparing glucocorticoid effects on lipolysis in subcutaneous and visceral adipose tissue depots, has not yet been reported.

Further *in vivo* support for an acute effect of cortisol on lipolysis comes from studies blocking glucocorticoid release. Plasma cortisol levels follow a diurnal rhythm, peaking 2-4 hours before wakening in humans, and reaching a nadir around midnight. Studies have confirmed that these physiological variations in cortisol influence fuel availability at a tissue level, by demonstrating reduced subcutaneous adipose tissue lipolysis and LPL activity (by A-V difference technique) following abolition of the morning rise in cortisol using metyrapone (Samra et al. 1996a). No effect of glucocorticoids on adipose tissue blood flow or FFA re-esterification rates was demonstrated (Samra et al. 1996a).

These results imply that acute elevations of cortisol within the normal physiological range can modulate adipose tissue function. In particular, effects on lipolysis may be most important when insulin levels are low, eg in patients with diabetes (Johnston et al. 1982;Schade et al. 1978) and when adrenaline levels are high. How this control is modulated in different adipose tissue depots remains, however, uncertain.

1.4.3.2 Chronic effects of glucocorticoids on adipose tissue and intravascular lipolysis

The above acute *in vivo* studies examined lipolysis in response to short term variations in cortisol levels lasting a number of hours. Few studies have examined the effects of prolonged administration of steroids, but these suggest that the acute induction of systemic lipolysis by glucocorticoids is not sustained, even if there may be ongoing enhanced lipolysis in subcutaneous adipose tissue. Gravholt *et al* found no change in systemic or leg adipose tissue lipolysis after one week of 30mg prednisolone/day, although subcutaneous abdominal adipose tissue lipolysis was increased (Gravholt et al. 2002). Miyoshi *et al* administered 50mg prednisolone/day for four days, and found no difference in rates of intracellular re-esterification or systemic lipolysis (Miyoshi et al. 1988). Similarly, Johnston *et al* found no difference in FFA concentrations, a surrogate marker of lipolysis, during partial insulin deficiency induced by somatostatin following 36-60 hours of 1mg tetracosactrin daily (Johnston et al. 1982).

Patients with Cushing's syndrome are heterogeneous in their characteristics and degree of glucocorticoid excess. However, consistent with the lack of effect of more than a few hours of glucocorticoid administration on systemic lipolysis in healthy volunteers, tracer studies reveal either unaltered or reduced systemic rates of lipolysis in patients with Cushing's syndrome when expressed as an absolute rate or relative to total body mass (Birkenhager et al. 1976;Saunders et al. 1980). However, in neither of these studies was the R_a of FFAs expressed relative to fat mass, or more specifically fat free mass (FFM), an indicator of resting energy expenditure (REE) considered by some authors to be the main determinant of FFA turnover. For example, an increase in FFA turnover in obesity is only measurable when it is expressed relative to FFM rather than total body mass (reviewed by (Koutsari and Jensen 2006)). Importantly, insulin levels are elevated in Cushing's patients, possibly suppressing any lipolytic tendency (Saunders et al. 1980). Subcutaneous adipose tissue lipolysis may be selectively increased in patients with Cushing's syndrome, at least when measured using microdialysis (Krsek et al. 2006), but biopsies of subcutaneous adipose tissue from such patients exhibit impaired lipolytic activity *in vitro* (Rebuffe-Scrive et al. 1988). Interestingly, LPL activity was increased in these biopsies when compared with both non-obese and obese controls, suggesting that contrasting regulation of LPL and intracellular lipolysis might explain some of the inconsistencies observed *in vivo*. However, LPL activity, as assessed by VLDL clearance, was unchanged following surgical treatment of Cushing's syndrome (Taskinen et al. 1983).

These somewhat inconsistent results suggest that chronic glucocorticoid excess is not associated with increased lipolysis measured at whole body level, although depot specific increases and compensatory decreases in lipolysis cannot be excluded.

1.4.3.3 Adipocyte differentiation and cell size

Compared to normal weight controls, individuals with Cushing's syndrome have an approximately two-fold and five-fold increase in subcutaneous and intra-abdominal (visceral) fat respectively, although limb subcutaneous adipose tissue may be

reduced, contributing the classical "lemon on sticks" description of Cushing's syndrome (Mayo-Smith et al. 1989). As well as regional differences in FFA metabolism, changes in adipocytes size and/or number may contribute to fat redistribution, with reduced adipocyte size seen in femoral adipose tissue in Cushing's disease (Rebuffe-Scrive et al. 1992;Rebuffe-Scrive et al. 1988). It is unclear whether these changes in fat distribution directly influence insulin resistance or metabolic fluxes to the liver.

1.4.3.4 Effects of glucocorticoids on de novo lipogenesis

Although effects on lipolysis have attracted most research effort, altered regional lipid synthesis could also influence fat redistribution following chronic glucocorticoid excess. Insulin is the main hormone promoting fatty acid synthesis and, in contrast to their apparently opposing effects on lipolysis, glucocorticoids may act synergistically with insulin to upregulate lipogenesis. Studies of adrenalectomised rats suggest that glucocorticoids are necessary for the lipogenic response to refeeding after starvation (Williams and Berdanier 1982). *In vitro*, glucocorticoids are necessary to potentiate the action of insulin on a number of lipogenic enzymes in rat hepatocytes (reviewed in (Hillgartner et al. 1995)), and synergism of insulin and dexamethasone has been shown in human adipocytes (Wang et al. 2004).

Most studies in humans have measured either whole body DNL indirectly using indirect calorimetry, or have specifically examined the contribution of hepatic DNL to VLDL secretion. Traditionally, DNL has been thought to make only a minimal contribution to adipose tissue TGs, with the majority of FFAs being derived from the diet, and until recently *in vivo* tracer studies examining DNL in adipose tissue have been lacking due to methodological considerations (Strawford et al. 2004). However, recent studies using prolonged (5-9 weeks) deuterated water administration suggest that approximately 20% of newly deposited adipose TGs are derived from DNL in non-obese subjects (Strawford et al. 2004). Further evidence of the importance of DNL is provided by recent findings in non-alcoholic fatty liver disease demonstrating that an increased proportion of hepatic TG is derived from hepatic

DNL and is associated with elevated rates of fasting DNL (Donnelly et al. 2005). Glucocorticoids increase rates of hepatic DNL contributing to VLDL, reducing the contribution from the stored cytosolic TG pool, thereby potentially contributing to hepatic steatosis and to increased export of TGs to adipose tissue depots (Dolinsky et al. 2004). Induction of DNL by glucocorticoids in selected adipose depots could also contribute to the obese Cushingoid phenotype, but has not yet been studied.

1.4.3.5 Effects of glucocorticoids on hepatic fatty acid metabolism

By contrast with the extensive literature describing effects of glucocorticoids on adipose tissue lipid metabolism, investigations in the liver have focused on effects on glucose rather than fat metabolism. *In vitro* studies suggest that glucocorticoids promote VLDL secretion possibly by increasing production and reducing degradation of apolipoprotein B (Wang et al. 1995). Accordingly, VLDL production rates are elevated in Cushing's syndrome, and since VLDL clearance is unaltered this accounts for increased circulating VLDL and atherogenic LDL levels (Taskinen et al. 1983). Additionally, glucocorticoids act to increase the activity of a number of enzymes involved in hepatic TG synthesis (Bates and Saggerson 1979;Lau and Roncari 1983;Pittner et al. 1985) which could promote steatosis.

1.4.3.6 Effects of glucocorticoids on fatty acid oxidation and energy expenditure

In order to preserve glucose for energy provision in the CNS, glucocorticoids might be expected to increase oxidation of FFAs in peripheral tissues. *In vitro* studies suggest that glucocorticoids have no direct effects on CPT1 activity, the essential rate limiting enzyme required for mitochondrial β-oxidation (Agius et al. 1986), although the capacity for mitochondrial β-oxidation in muscle may be increased (Short et al. 2004), and peroxisomal β-oxidation may be increased in the liver (Norrheim et al. 1990). However, there is also some *in vitro* data to suggest that glucocorticoids may inhibit FFA oxidation (Letteron et al. 1997). *In vivo* studies of effects of glucocorticoids on β-oxidation have been inconclusive; these studies are problematic as altered substrate availability and compensatory hormonal changes may also influence fatty acid oxidation. For example, in one study lipid oxidation, measured

by indirect calorimetry, was augmented by hypercortisolaemia, but this may have been secondary to increased Ra of FFAs (Djurhuus et al. 2002).

Both resting energy expenditure (REE) and lipid oxidation have been shown to be unaltered in Cushing's syndrome and unchanged after successful treatment (Burt et al. 2006;Burt et al. 2007), whereas exogenous glucocorticoids may actually slightly increase REE (Brillon et al. 1995;Chong et al. 1994), in contrast to their effects to reduce thermogenesis in rodents (Soumano et al. 2000).

1.4.3.7 Appetite and food selection

Central effects of glucocorticoids on appetite are well known (reviewed in (Dallman et al. 2004)), and increased energy intake could clearly contribute to glucocorticoid induced weight gain. Tataranni *et al* demonstrated increased food intake in response to glucocorticoids in humans, greatly in excess of a slight increase in energy expenditure (Tataranni et al. 1996). Stimulation of appetite by glucocorticoids occurs despite elevations in the satiety hormone leptin (Udden et al. 2003). Although Tatarinni's study suggested that glucocorticoids mainly stimulate protein and carbohydrate intake in humans (Tataranni et al. 1996), studies in adrenalectomised and streptozotocin treated rodents suggest that insulin is key in influencing food choice and increasing fat intake in response to glucocorticoids (la Fleur et al. 2004). Thus, again prevailing insulin concentrations may be important in determining the response to glucocorticoids, suggesting the hyperinsulinaema associated with insulin resistance may be influencial.

1.4.4 Glucocorticoid treatment and NAFLD in humans

It is unclear whether the above changes in fatty acid metabolism contribute to the development of NAFLD in humans. There is a pausity of clinical data to support the hypothesis that chronic glucocorticoid therapy leads to steatosis, with only 20% of patients with Cushing's syndrome having NAFLD, albeit with non-gold standard imaging (CT) (Rockall et al. 2003). One case series of patients with systemic lupus erythematosis (SLE), a connective tissue disease, not infrequently requiring treatment with glucocorticoids, found 73% of patients to have evidence of fatty liver

on liver histology (Matsumoto et al. 1992), with an association between total prednisolone dose and the severity of fatty liver. In a subsequent series, of liver biopsy and autopsy findings in SLE patients with high and low glucocorticoid exposure, only high total steroid dosage associated with severe steatosis, with 6% having evidence of steatohepatitis (Matsumoto et al. 2007). However, clearly these cases series have multiple confounders, most notably, the underlying severity of the SLE and the case selection bias.

Other limited evidence includes a few isolated cases reports of glucocorticoid induced steatohepatitis, (reviewed in (Matsumoto et al. 2007)), including cases with both acute and chronic glucocorticoid exposure. Perhaps for this reason, as well as the concern over exacerbating any associated metabolic abnormalities and steatosis, there have been no therapeutic trials of steroids in NASH to date, despite their apparent success in treating acute alcoholic steatohepatitis (Forrest et al. 2007).

1.4.5 Glucocorticoids and inflammation

HPA axis activation, leading to increased glucocorticoid production, is an essential component of the stress-inflammatory response, as evidenced in patients with adrenal insufficiency in whom infection is life threatening (Kapcala et al. 1995). However, chronic overactivity e.g. in Cushing`s syndrome leads to immunosuppression and increased susceptibility to infection (Lionakis and Kontoyiannis 2003), suggesting glucocorticoids have a complex role in modulating the inflammatory/immune response.

In brief, inflammation can be considered the tissue response to injury, and can be both acute and chronic. In general, acute inflammation involves the innate immune system and acts to enhance healing and protect against spread of pathogens, whereas chronic inflammation involves the more specific adaptive immune system and can lead to complications such as tissue damage and fibrosis.

Acute injury leads to a pro-inflammatory response from resident cells producing histamine and cytokines such as TNFα and IL-1 thought to be important in the development of NASH (Koppe et al. 2004). This leads to an increase in vascular permeability and leukocyte migration, contributing to the classical signs of swelling, redness, heat and pain. Resolution of acute inflammation involves phagocytosis of the acute inflammatory infiltrate (mainly apoptotic neutrophils) by macrophages (Coutinho and Chapman 2010). Possible delays in this mechanism may lead to phagocytic consumption of necrotic versus apoptotic neutrophils which, coupled with ongoing toxic insults may promote a chronic inflammatory response (Botto et al. 1998). Chronic inflammation is characterised by the accumulation of activated macrophages which may promote tissue damage through the release of pro-inflammatory and pro-fibrotic mediators (Coutinho and Chapman 2010).

1.4.5.1 Glucocorticoids and the resolution of inflammation

The term immunomodulatory is often used to describe glucocorticoids` effects on the immune system which are permissive, stimulatory, and suppressive (Yeager et al. 2004). The balance of these effects is likely to depend on the concentration of endogenous and/or exogenous glucocorticoids available as well as timing in relation to the onset of inflammation.

The glucocorticoid receptor is almost ubiquitously expressed and many aspects of the inflammatory and immune response are regulated by glucocorticoids. Firstly, they inhibit prostaglandin production by a number of mechanisms including decreasing cyclo-oxygenase 2 expression, indirectly inhibiting phospholipase A2 production, and by blocking the MAPK pathway by increasing MAPK phosphatase 1 (Rhen and Cidlowski 2005). Glucocorticoids also inhibit NFκB a key transcription factor regulating pro-inflammatory cytokine and prostaglandin production (McKay and Cidlowski 1999), and influence gene transcription of a number of transcription factors for pro-inflammatory cytokines and their receptors, whilst increasing expression of anti-inflammatory cytokines e.g. IL-10 (Barnes 1998). NFκB is also upregulated by FFAs and is thought to be a key factor in the development of NASH (Boden et al. 2005).

Numerous other effects of glucocorticoids may be relevant in NASH including effects on leucocyte trafficking, and cellular differentiation, as well as gene transcription in monocytes/macrophages, neutrophils and granulocytes (Coutinho and Chapman 2010). In particular glucocorticoids increase the phagocytic capacity of macrophages, and promote an anti-inflammatory phenotype in differentiating monocytes/macrophages (Giles et al. 2001). More rapid non-genomic effects of glucocorticoids may also influence the development of inflammation including effects on vasodilatation (Hafezi-Moghadam et al. 2002). In general glucocorticoids tend to promote the resolution of inflammation, suppressing the acute inflammatory response and would be predicted to reduce the development of steatohepatitis, albeit that this may be offset by the metabolic cosequences of increased glucocorticoids.

1.4.5.2 11β-HSD1 and inflammation

In addition, alterations in local 11β-HSD1 activity may also provide additional "fine tuning" and amplification of tissue glucocorticoid concentrations in response to injury, and has been shown to influence the inflammatory response in a number of animal models (Coutinho and Chapman 2010).

The influence of 11β-HSD1 on amplifying local glucocorticoid signalling has been discussed in Section 1.4.1. 11β-HSD1 is present in most immune cells and its expression increases on activation of macrophages, and T and B lymphocytes (Zhang et al. 2005). Its activity is upregulated by the pro-inflammatory cytokines TNFα and IL-1 (Escher et al. 1997), as well as the Th2 cytokines IL-4 and IL-13 (Thieringer et al. 2001).

Studies examining the inflammatory response e.g. thioglycollate induced sterile peritonitis in 11β-HSD1 knockout mice and using 11β-HSD1 inhibitors in cell lines, demonstrate that 11β-HSD1 plays an important role in suppressing pro-inflammatory cytokines and increasing phagocytic activity of macrophages, promoting the resolution of inflammation (Gilmour et al. 2006;Ishii et al. 2007). Similarly, 11β-

HSD1 inhibition reduced the macrophage chemoattractant protein MCP-1 levels in *ApoE*-/- mice (Hermanowski-Vosatka et al. 2005).

In addition to effects on inflammatory cells, pro-inflammatory cytokines induce 11β-HSD1 activity in a number of other cells types including adipocytes, osteoblasts, mesangial cells, synovial fibroblasts, vascular smooth muscle, and both ovarian granulosa and surface epithelial cells (reviewed in (Chapman et al. 2009)). More recent work has focussed on the role of 11β-HSD1 in modifying the vascular inflammatory response (Hadoke et al. 2009).

Interestingly studies have shown increased 11β-HSD2 activity in macrophages from patients with rheumatoid arthritis, possibly hampering anti-inflammatory effect of glucocorticoids in this chronic inflammatory disease, and adding further complexity to the regulation of glucocorticoid metabolism in inflammation (Hardy et al. 2008).

1.4.6 Glucocorticoids and fibrosis

It follows from the above that glucocorticoids have a myriad of effects on the immune system that could suppress the development of inflammation and fibrosis in NAFLD, although with the risk this could be offset by their metabolic side effects. I will briefly discuss the evidence that glucocorticoids influence hepatic fibrogenesis.

In addition to their anti-inflammatory effects, glucocorticoids are able to reduce type 1 collagen synthesis, the final common pathway in fibrogenesis, by interfering with TGFβ signalling (Cutroneo and Sterling, Jr. 2004). Type III collagen is also important in the wound healing response and its expression is reduced by glucocorticoids, associated with a decrease in *Timp1* and *Timp2* expression, in the skin at least (Oishi et al. 2002). Additional results from cultured hepatic stellate cells confirm that glucocorticoids interrupt TGFβ signalling and secretion (Bolkenius et al. 2004). Furthermore, glucocorticoids have been shown to reduce TGFβ induced apoptosis, a possible driver of the fibrotic process, in a hepatoma cell line (Evans-Storms and Cidlowski 2000;Wanke et al. 2004). Another potential antifibrotic

mechanism of glucocorticoids may relate to reducing angiotensin 1 receptor expression in hepatoma cells (Wintersgill et al. 1995).

As discussed, carbon tetrachloride (CCl_4) injury and common bile duct ligation are commonly employed models of liver fibrosis and have been used to confirm the antifibrotic effects of glucocorticoids e.g. glucocorticoids have been shown to reduce the extent of fibrosis following bile duct ligation (Eken et al. 2006) and the glucocorticoid antagonist RU38486 increased acute CCl_4 induced liver injury, associated with a reduction in hepatic TGs, the former possibly via reduced *IL-10* expression (Swain et al. 1999). However, selective delivery of dexamethasone to Kupffer cells actually produced an increase in fibrosis and *Timp1* expression, desite a reduction in ROS production, suggesting the site of glucocorticoids actions are essential in determining their effects (Melgert et al. 2001).

1.4.7 Abnormalities of glucocorticoid metabolism and NAFLD

The above metabolic and anti-inflammatory effects of glucocorticoids emphasise their potential role in the pathogenesis of NAFLD. Although circulating glucocorticoid levels are not abnormal in NAFLD, local glucocorticoid actions on the liver are determined by hepatic glucocorticoid metabolism, and a number of findings suggest that abnormalities of glucocorticoid metabolism may be important in relation to NAFLD. These will discussed below.

1.4.7.1 Altered glucocorticoid metabolism and NAFLD

Mice overexpressing *11β-HSD1* in adipose tissue (under the aP2 promoter) develop central obesity, hypertension, dyslipidaemia, and glucose intolerance (Masuzaki et al. 2001;Masuzaki et al. 2003), whereas mice overexpressing *11β-HSD1* in the liver (under the ApoE promoter) develop insulin resistance and hypertension but not obesity (Paterson et al. 2004). Both have increased liver TGs. Conversely, *11β-HSD1* null mice are protected from the metabolic syndrome, resisting hyperglycaemia and obesity induced by a high fat diet, with redistribution of fat in favour of subcutaneous depots (Kotelevtsev et al. 1997;Morton et al. 2004). In addition to effects on

glucose/insulin homeostasis, these tissue-specific manipulations of *11β-HSD1* in mice are associated with a host of alterations in fatty acid metabolism. aP2 *11β-HSD1* overexpressors have a three fold increase in plasma FFA (as well as corticosterone) levels in the portal vein, with a less pronounced elevation in the systemic circulation (Masuzaki et al. 2001). Serum TG levels are also increased. ApoE *11β-HSD1* overexpressors have increased serum FFAs on a low fat diet (Paterson et al. 2004). In addition, hepatic gene expression studies suggest that both fatty acid synthesis and lipid oxidation is basally upregulated, although the lipogenic response to high fat feeding was attenuated compared to controls (Paterson et al. 2004). In contrast, *11β-HSD1* null mice have a favourable plasma lipoprotein profile with increased hepatic insulin sensitivity, but also an apparent increase in hepatic lipid oxidation as measured by gene expression studies (Morton et al. 2001). These findings suggest that variations in glucocorticoid concentrations selectively within either the adipose tissue or the liver influence FFA metabolism and hepatic TG accumulation.

Pharmacological inhibition of 11β-HSD1 as a novel therapy for type 2 diabetes is a current goal for a number of pharmaceutical companies, with promising results for glucose/insulin homeostasis in animal models (reviewed in (Stimson and Walker 2007)) and now humans (Rosenstock et al. 2010). In rats fed a high fat diet an 11β-HSD1 inhibitor reduced serum FFA levels, reduced mesenteric adipose tissue weight, decreased adipose tissue expression of genes involved in lipid synthesis and FFA/TG cycling (lipolysis and re-esterification), and increased those involved in lipid oxidation (Berthiaume et al. 2007a). Activity of key lipogenic enzymes in the liver was unaltered by 11β-HSD1 inhibition but fasting serum TG levels were reduced through reduced VLDL secretion (Berthiaume et al. 2007b). Uptake and oxidation of TG derived FFA appeared to be increased in peripheral tissues, but interestingly muscle LPL activity was not increased (Berthiaume et al. 2007b). 11β-HSD1 inhibitors also improve the serum lipoprotein profile and reduce the progression of atherogenesis (Hermanowski-Vosatka et al. 2005).

Inactivation of other glucocorticoid metabolising enzymes can also influence the development of NAFLD. Inactivation of the predominantly hepatic isoform of 5α–reductase (type 1), predicted to increase intrahepatic glucocorticoid levels, leads to fatty liver and insulin resistance (Livingstone et al. 2009b).

These findings demonstrate that transgenic and pharmacological modulation of glucocorticoid metabolism can influence systemic and hepatic fatty acid metabolism leading to the development of NAFLD, but what is the evidence that abnormalities of glucocorticoid metabolism exist in NAFLD?

1.4.7.2 NAFLD and alterations in glucocorticoid metabolism

Obese individuals have increased 11β-HSD1 mRNA transcripts and enzyme activity in subcutaneous adipose tissue *in vitro* (Kannisto et al. 2004;Lindsay et al. 2003;Rask et al. 2001;Wake et al. 2003) and increased rates of cortisol regeneration in subcutaneous adipose tissue *in vivo* (Sandeep et al. 2005). In contrast, 11β-HSD1 activity is reduced in the liver in obesity (Rask et al. 2001;Stewart et al. 1999), with upregulation of 5α- and 5β-reductases (Andrew et al. 1998). Whether 11β-HSD1 is also up-regulated in visceral adipose tissue in obesity is uncertain, with some studies showing increased activity (Desbriere et al. 2006;Michailidou et al. 2007) and others showing no change (Alberti et al. 2007;Goedecke et al. 2006;Tomlinson et al. 2002), although most recent evidence suggests there is negligible visceral adipose cortisol production (Stimson et al. 2009).

Similar changes in hepatic glucocorticoid signalling have been found in rodent models of obesity including Zucker obese rats (Livingstone et al. 2005;Livingstone et al. 2000a) and high fat fed rats (Drake et al. 2005). These models develop steatosis and have upregulation of 5α- and 5β-reductases associated with downregulation of hepatic 11β-HSD1, predicting increased clearance of glucocorticoids, and reduced intrahepatic glucocorticoid levels, although compensatory HPA axis activation may act to maintain circulating glucocorticoid concentrations. These changes in hepatic metabolism may therefore be acting to protect the liver from metabolic consequences of GR activation.

Limited studies in humans have provided conflicting results, perhaps reflecting the heterogeneity of patient groups studied. In a study of non-obese (mean BMI 26.4) individuals with increased liver fat, subcutaneous and hepatic 11β-HSD1 activity were not altered, whereas intrahepatic 5β-reductase activity was elevated (Westerbacka et al. 2003). In a more recent study, currently only published in abstract form, patients with biopsy proven NAFLD had increased 5α-reductase activity, with reduced hepatic 11β-HSD1 activity, thus seeming to limit hepatic glucocorticoid exposure. Total glucocorticoid production rate was also increased, consistent with HPA axis activation. However, in the group of patients with steatohepatitis, hepatic *11β-HSD1* mRNA transcripts and immunohistochemical staining was markedly increased (Ahmed et al. 2010). In contrast, another recent human study of 75 patients found no association with 11β-HSD1 activity and NASH, whilst 5α and 5β-reductase activity was increased in patients with NAFLD with and without NASH (Konopelska et al. 2009).

In keeping with increased clearance of glucocorticoids, a number of clinical studies have now suggested overactivation of the HPA axis in NAFLD (Targher et al. 2005;Westerbacka et al. 2003;Zoppini et al. 2004), which may increase adrenal androgen production (Saruc et al. 2003). Furthermore, in one study 24 hour urinary free cortisol concentrations and post dexamethasone suppression cortisol levels strongly correlated with the degree of hepatic inflammation and fibrosis in NAFLD, although abnormal metabolism of dexamethasone in NAFLD is a potential confounder (Targher et al. 2006). Nevertheless, increased glucocorticoid clearance would be predicted to reduce intrahepatic glucocorticoid levels, potentially exacerbating hepatic inflammation in NAFLD.

It is noteworthy that 5β-reductase is also responsible for the synthesis of bile acids from cholesterol (Danielsson and Sjovall 1975). Bile acids, especially hydrophobic ones such as CDCA, are inherently toxic to hepatocytes, and alterations in bile acid metabolism may have implications for the pathogenesis of NASH.

Lastly, it is possible that differences in glucocorticoid signalling in metabolic syndrome and NAFLD are a consequence rather than cause of variations in fatty acid metabolism. Although they do not appear to influence cortisol and ACTH secretion (Mai et al. 2006), plasma FFAs and dietary macronutrient content have been shown to influence local glucocorticoid metabolism in some (Stimson et al. 2007;Wake et al. 2006) but not all human studies (Mai et al. 2005). There are also reports that FFAs alter GR function (Sumida 1995). However, the most recent evidence supports the concept that variations in 11β-HSD1 influence fatty acid metabolism.

1.5 HYPOTHESIS AND AIMS

There is increasing evidence that abnormalities of peripheral and intrahepatic fatty acid metabolism may contribute to both the development and progression of non-alcoholic fatty liver disease. In particular, factors promoting the accumulation of intrahepatic fatty acids may promote progression to steatohepatitis and fibrosis, and influence the metabolic complications of NAFLD. In addition, abnormalities of glucocorticoid metabolism have been implicated in the development of the metabolic complications of obesity, including NAFLD. The liver plays a central role in regulating glucocorticoid metabolism which, given the metabolic and anti-inflammatory properties of glucocorticoids, make it vulnerable to changes in local glucocorticoid levels. It is not clear to what extent local changes in hepatic glucocorticoid metabolism, e.g. those predicted to reduce intrahepatic glucocorticoid levels, contribute to the development of steatosis, steatohepatitis and/or liver fibrosis. This is important as inhibitors of 11β-HSD1 may be introduced as a treatment for type 2 diabetes, a condition associated with NAFLD.

The hypotheses in this PhD were as follows:

1) by studying dietary models of NAFLD in rodents in which the risk of insulin resistance, steatohepatitis and fibrosis differs, one can identify pathways that play a key role in determining these risks.

2) altered patterns of fatty acid metabolism, predicted to increase intrahepatic fatty acids, associate with patterns of risk in NAFLD and increase susceptibility to steatohepatitis and fibrosis.

3) altered glucocorticoid metabolism, with reduced 11β-HSD1 activity or increased A-ring reductase activity, predicted to reduce intrahepatic glucocorticoid levels, associates with patterns of risk in NAFLD and increases susceptibility to steatohepatitis and fibrosis.

To address the above hypotheses, I undertook studies with the following aims:

1) in **Chapter 3**, I investigated the susceptibility to steatohepatitis and carbon tetrachloride (CCl_4) induced liver fibrosis in choline deficient diet (CDD) and methionine and choline deficient diet (MCDD) models of fatty liver in mice to address the following:

a) To confirm whether a MCDD produces significantly greater steatohepatitis than CDD in mice
b) Whether steatohepatitis in MCDD is associated with upregulation of pro-inflammatory cytokines
c) Whether steatohepatitis in MCDD mice increases susceptibility to CCl_4 induced hepatic fibrosis
d) Whether susceptibility to liver fibrosis is associated with downregulation of specific matrix metalloproteinases (MMPs) and/or upregulation of their inhibitors (TIMPs)

2) in **Chapter 4**, I investigated *in vivo* fatty acid metabolism in CDD and MCDD models of fatty liver, using a number of methodologies, to address the following:

a) whether weight loss in MCDD, associated with steatohepatitis, is a result of increased peripheral lipolysis increasing the flux of fatty acids to the liver, or impaired export of fatty acids from the liver, reducing the supply of triglycerides to adipose tissue
b) whether increased hepatic de novo lipogenesis, predicted to increase intrahepatic fatty acids, is associated with steatohepatitis in MCDD mice

3) in **Chapter 5**, I investigated glucocorticoid metabolism in CDD and MCDD mice treated with carbon tetrachloride to induce liver fibrosis, to establish the likely contribution to NAFLD, NASH and fibrosis severity. Real time PCR studies and enzyme activity assays were performed to address the following:

a) whether differences in hepatic glucocorticoid metabolism associate with differing susceptibility to steatohepatitis in CDD and MCDD fed mice
b) whether impaired 11β-HSD1 activity, predicted to reduce intrahepatic glucocorticoid levels, associates with susceptibility to steatohepatitis and/or fibrosis in MCDD mice
c) whether increased clearance of glucocorticoids by A-ring reductases, predicted to reduce intrahepatic glucocorticoid levels, associates with susceptibility to steatohepatitis and/or fibrosis in MCDD mice

Finally in **Chapter 6**, I discuss the significance of the above results and their relevance for the development of progressive non-alcoholic fatty liver disease in humans.

2 Chapter 2-Materials and Methods

2.1 MATERIALS

All chemicals were from Sigma (Poole, UK) unless otherwise stated.
Solvents were glass-distilled HPLC grade (Rathburn, Walkerburn, UK).
Stable-isotope labelled tracers (clinical grade) were from Cambridge Isotopes (Andover, MA, USA).
Radioactively labelled steroids were from Amersham (Buckinghamshire, UK).
Human albumin solution (20%w/v) was from the Scottish National Blood Transfusion Service (Lothian, UK).
All sterile needles (Microlance) and syringes (Plastipak) were from Becton Dickinson (Oxford, UK).
Pyrex glass reaction tubes and screw top vials were purchased from Fisher Scientific (Loughborough, Leicestershire, UK).
Room temperature was 22+/-2^{o}C.

2.2 ANIMALS

All experiments were carried out under a UK Home Office animal license.
C57BL/6J mice (Harlan, Bicester, UK) were maintained under controlled conditions of light and temperature. Mice were housed in a 12 hour light (07.00h to 19.00h) 12 hour dark cycle, with a temperature range of 22+/-2^{o}C. Diets were from Dyets Inc. Bethlehem, PA), and the dietary compositions are shown in Table 2.1; to prevent unavoidable contamination of choline and methionine from protein, the protein content was replaced by pure *L*-amino acids (Nakae 1999).

Table 2.1 Composition of rodent diets

Diets were purchased from Dyets Inc (Bethlehem, PA), with reference codes in brackets. CS; (518574), CDD; (518753), and MCDD; (518810). All diets contained 50g/kg corn oil (source of polyunsaturated fat) and 100g/kg Primex (hydrogenated vegetable oil) as lipid sources. Carbohydrate was composed of corn starch, dextrin, cellulose, and sucrose, the latter differing marginally between the groups (392, 406 and 408g/kg for the CS, CDD and MCDD, respectively).

	Control diet (CS)	**Choline deficient diet (CDD)**	**Methionine and choline-deficient diet (MCDD)**
Kcal/gram	4.3	4.3	4.3
Kcal%			
Protein (as *L*-amino acids)	15	15	15
Carbohydrate	55	55	55
Fat	30	30	30
Choline Bitartate (g/kg)	14.48	0	0
L-Methionine (g/kg)	1.7	1.7	0

2.3 COMMONLY USED SOLUTIONS

TBAHS buffer: dibasic potassium phosphate (0.2M) & tetrabutylammonium hydrogen sulphate (0.05M) adjusted to pH 9.0 with potassium hydroxide (1M) and stored at 4^{o}C.

Iodomethane solution: Iodomethane in dichloromethane (1:10, v/v), prepared daily.

Chloroform-methanol solution: (2:1, v/v) containing 50µg/ml butylated hydroxytoluene.

Stock solutions: palmitate (1µg/µl in chloroform), 7,7,8,8-$^{4}[H_2]$ palmitate (D4 palmitate) (1µg/µl in chloroform) and heptadecanoic acid (internal standard, 1µg/µl in chloroform) were stored at -80^{o}C.

Citric acid buffer: 0.01M citric acid buffer adjusted to pH 6.0 with 1M sodium hydroxide.

DEPC treated water: Distilled water (500ml) mixed with 500µl (0.1% v/v) diethyl pyrocarbonate (DEPC). The solution was allowed to stand for 12-24 hours before being sterilised by autoclave.

10x TBE buffer: 108g Tris-base, 55g boric acid, 40ml 0.5M EDTA (pH 8.0), adjusted to 1L with distilled water. The solution was sterilised by autoclave before use.

KREBS buffer: 118mM NaCl, 3.8mM KCL, 1.19mM KH_2PO_4, 2.54mM $CaCl_2$, 1.19mM $MgSO_4$, 25mM $NaHCO_3$ (all sterilised by autoclave before use), adjusted to 1L with ultrapure water (Milli-Q Academic system, Millipore, Watford, UK) adjusted to pH 7.4 with 5M HCl. The solution was stored at 4ºC.

2.4 INTRODUCTION TO METHODS

The majority of the assays and techniques had previously been used in our laboratory and I subsequently learned the methods as required. Some of the techniques had not previously been employed: e.g. the use of stable isotope metabolic tracer techniques in mice requiring fatty acid measurement by gas chromatography mass spectrometry; mass isotopomer distribution analysis of labelled dietary acetate; solid phase extraction of plasma and tissue lipids; jugular venous cannulation in mice; and administration of intravenous tyloxapol to measure VLDL export. I undertook development of these new techniques and methods.

2.5 HISTOLOGY AND IMMUNOHISTOCHEMISTRY

2.5.1 Introduction

In Chapters 3 and 4 sections of liver were stained using routine tinctorial stains and immunohistochemistry undertaken as described below.

2.5.2 Tinctorial stains

Sections of liver were fixed in 10%v/v formalin solution for 12 hours prior to embedding in paraffin. ~5µm sections of the paraffin embedded tissue were cut using a microtome and stained with sirius red, and haematoxylin and eosin by the University of Edinburgh histology department as follows.

2.5.2.1 Haematoxylin and eosin

Routine staining with haematoxylin and eosin (H&E) staining enables gross visualisation of hepatic architecture, staining nuclei blue and matrix pink. Liver sections were dewaxed in xylene for 10mins followed by rehydration through alcohols (5mins each in 100%, 100%, 70%, 50%v/v ethanol). Sections were then placed in Harris' haematoxylin for 30 seconds, washing in running tap water (~60secs) and rinsed with Scott's tap water (~5secs). After a further wash in tap water sections were then differentiated in acid-alcohol (3secs) and staining in eosin (60secs). Sections were then dehydrated in increasing concentrations of alcohols, cleared in xylene and mounted using DPX.

2.5.2.2 Picosirius red

Liver sections were dewaxed and rehydrated as described above. They were then stainined in picosirius red/fast green solution for two hours. After a brief wash in running tap water the sections were dehydrated in increasing concentrations of alcohol, cleared in xylene and fixed as before.

2.5.3 Immunohistochemical staining

The immunohistochemical staining of liver sections for alpha smooth muscle actin (a marker of activated hepatic stellate cells), macrophages and neutrophils is described. These techniques were used in Chapters 3 and 4.

2.5.3.1 Alpha smooth muscle actin

For immunostaining for alpha smooth muscle actin (αSMA), liver sections were de-waxed in xylene and rehydrated in alcohol. Antigen retrieval was achieved by microwaving in 0.01M citric acid buffer (pH 6.0). Endogenous peroxidase activity was blocked by washing in 1%v/v hydrogen peroxide solution, and slides mounted in Shandon Sequenza racks (Thermo Fisher Scientific Inc, Leics, UK), after washing in phosphate buffered saline (PBS)**.** A commercial avidin/biotin blocking kit (Vector Labs, Burlingame, CA, USA) was used to block any background staining, washing with PBS (x3) between stages. Samples were further blocked with MOM mouse immunoglobulin blocking agent (Vector Labs, Burlingame, CA, USA) for 60mins. Sections were then incubated overnight at 4ºC with a 1 in 6000 dilution of the primary αSMA antibody (A5228 monoclonal mouse anti αSMA, Sigma, UK) prepared in MOM diluent. After 0.1%v/v tween and PBS washes, sections were incubated with the biotinylated secondary antibody (MOM biotinylated anti-mouse IgG reagent, Vector Labs, Burlingame, CA, USA) for 10mins at room temperature. After additional tween and PBS washes, sections were incubated with 3 drops of Vector RTU ABC (Avidin and Biotin horseradish peroxidase macromolecular Complex) reagent for 30mins at room temperature. Cells positive for αSMA were visualised by diaminobenzidine (DAB) staining. Sections were counterstained in haemotoxylin (~15secs), and rinsed in Scott`s tap water (~5secs) prior to dehydration through alcohol, clearing in xylene and mounting in DPX mountant.

2.5.3.2 Macrophage staining

Immunostaining for hepatic macrophages was performed using an anti F4/80 antibody (Ab6640 rat monoclonal anti F4/80 antibody, Abcam, Cambridge, UK). Sections were processed as described above up until and including the biotin blocking stage, except that no antigen retrieval step was required. Sections were blocked using normal rabbit serum (DAKO, Ely, UK) diluted 1:4 in PBS, incubating for 30mins at room temperature. Samples were then incubated with the primary anti F4/80 antibody, diluted 1in 300 in rabbit serum (60mins at room temperature). After further washes, sections were incubated with a 1:300 dilution of the secondary rabbit anti-rat antibody (DAKO) for 30mins at room temperature. Sections were incubated

with 3 drops of the Vector RTU ABC reagent (30mins at room temperature) and F4/80 positive cells were then visualised using DAB as before and samples counterstained and dehydrated as described above.

2.5.3.3 Neutrophil staining

Immunostaining for neutrophils was identical until the incubation with a 1 in 200 dilution (in rabbit serum) of the primary anti-granulocyte antibody (108413 Rat anti-mouse GR1 antibody, Cambridge Biosciences, Cambridge, UK) overnight at 4ºC. After 0.1%v/v tween and PBS washes, sections were incubated for 60mins at room temperature with a 1:300 dilution (in normal rabbit serum) of the secondary rabbit anti-rat antibody (DAKO). Processing then continued as above using the Vector ABC reagent and DAB, before dehydrating and mounting.

2.6 MESSENGER RNA (mRNA) EXTRACTION AND QUANTIFICATION

Gene expression studies, quantifying mRNA transcription in murine livers, were undertaken in Chapters 3 and 5.

2.6.1 RNA extraction

Total hepatic RNA was extracted from frozen liver using a Qiagen RNeasy extraction kit (Crawley, UK) as per the manufacturer's instructions. 20-30mg of liver was homogenised in 600µl RLT buffer (Qiagen, Crawley, UK) prior to centrifuging at 12,000 x g for 3mins at 4ºC. The supernatant was then added to 700µl 50% ethanol, and 700µl of the mixture added to the RNeasy mini column, prior to centrifugation (as above) for 15secs. The eluate was discarded and the remainder of the ethanol containing mixture passed through the mini columns and discarded. Columns were washed with further buffers (RWI and RPE, Qiagen, Crawley, UK) and centrifuged as above for 15 seconds. A final RPE wash was undertaken followed by centrifugation as above for 2mins, followed by elution of the RNA into RNAase free

water (30µl). Samples were split into aliquots to reduce the need for repeat thawing, and stored at -80ºC.

2.6.2 RNA quantification and integrity

The integrity of extracted RNA was assessed by agarose gel electrophoresis. After cleaning the tank with RNase Away solution (Molecular BioProducts, CA, USA), a 1.2%w/v agarose (Lonza, Basel, Switzerland) gel was prepared using 0.5%w/v tris borate EDTA (TBE) buffer and 10µl/100ml SYBR safe DNA stain (Invitrogen, Paisley, UK). For each sample, 1µl of extracted RNA solution was added to 10µl RNAase free water, and 2µl of loading dye, and loaded into wells. Electrophoresis was performed in 0.5%w/v TBE buffer at 100V for approximately 60mins. The separated 28S and 18S ribosome bands were then visualised at 254nm, using a UVIpro system (UVItec, Cambridge, UK), expecting an approximate 2:1 ratio if RNA integrity maintained.

Total RNA was quantified by measuring ultraviolet absorbance at 260nm (RNA) and 280nm (protein) by spectrophotometry (NanoDrop 1000, Thermoscientific) using 2µl of RNA solution loaded onto the spectrophotometer pedestal, after calibration using a 2µl RNAase free water 'blank'. A measured RNA concentration >100ng/µl, and an A260/A280 ratio >1.8 was deemed acceptable (pure RNA has a ratio of 2.1). To ensure similar quantities of RNA were reverse transcribed, aliquots of RNA were diluted with RNAase free water to achieve a concentration of 100ng/µl.

2.6.3 Synthesis of complemetary DNA (cDNA) by reverse transcription

A commercial reverse transcription kit (Qiagen, Cawley, West Sussex, UK) was used to eliminate genomic DNA contamination and then reverse transcribe mRNA (0.5µg) to cDNA. Genomic DNA 'wipeout' was undertaken using a reaction mixture containing the mRNA (5µl), water (7µl) and gDNA wipeout buffer (2µl), incubating at 42ºC for 2mins, followed by cooling to 5ºC. Reverse transcriptase (1µl), 5x RT buffer, containing 12.5mM $MgCl_2$ (4µl), and primer mix (1µl) was added to each

RNA sample creating a 20µl reaction mixture. Control samples were also prepared, except either the reverse transcriptase (-RT control), or RNA (negative template control), was substituted with water. Reverse transcription was performed using a thermal cycler (G-storm 1, Genetic Research Instrumentation Ltd, Essex, UK) programmed to 42ºC for 30mins (reverse transcription), 95ºC for 5mins (enzyme inactivation) and 4ºC for 5mins (to prevent reannealing). cDNA was stored at -20ºC until analysis.

2.6.4 Real Time Polymerase Chain Reaction (PCR)

Quantification of hepatic gene transcripts was performed by real time PCR using the Roche Lightcycler 480 system (Burgess Hill, UK). Primers and probes were designed from the Roche UPL library or were purchased pre-made (TaqMan Gene Expression Assays, Applied Biosystems, Southhampton, UK). Synthesised primers (Invitrogen, Paisley, UK) were dissolved in Tris EDTA buffer to achieve stock solutions of 100µmol/l.

cDNA from each sample was diluted 1:40 in nuclease free water. 10µl reaction volumes were loaded on to 384 multi-well PCR plates, consisting of cDNA (2µl), Lightcycler 480 RNA master hydrolysis probes mix (Roche Diagnostics, West Sussex, UK) (5µl), forward primer (0.1µl of 1:5 dilution of stock solution), reverse primer (0.1µl of 1:5 dilution), probe (Roche Diagnostics, West Sussex, UK) (0.1µl) and nuclease free water (2.7µl). The PCR plates were sealed and centrifuged at 2000 x g for 1min at 4ºC for 2minutes, to ensure adequate mixing. The Lightcycler was programmed as per the manufacturer's instructions: 95ºC for 5mins (DNA polymerase activation), 95ºC for 10secs (denaturation), and 60ºC for 30secs (annealing and extension), repeating the latter two stages for 40 cycles.

The hydrolysis probes contain a fluorescent dye reporter (FAM) and closely linked quencher (TAMRA). The Taq DNA polymerase degrades the annealed probe releasing the fluorescent dye, proportional to the amount of accumulating PCR product, enabling quantification. Fluorescence was monitored during each PCR cycle,

at 483nm and 533nm, corresponding to the excitation and emission wavelengths of the probes.

Seven point standard curves were created by pooling equal volumes of cDNA from each sample, and serial dilutions (1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512) prepared with nuclease free water. Samples were analysed in triplicate and the second derivative maximum or crossing point (Cp) method used for construction of the standard curve and relative quantification of PCR product. The variability of triplicates was considered acceptable if standard deviation was <0.5, and a standard curve efficiency of >1.7 was deemed acceptable. Transcript levels were normalised to abundance of transcripts of housekeeping, internal control genes.

2.7 BACKGROUND TO METABOLIC TRACER TECHNIQUES

In Chapter 4 I undertook detailed physiological studies of fatty acid metabolism in mice which included the use of stable isotope tracers to quantify rates of fatty acid turnover and synthesis. In the following section I will discuss the background to these techniques followed by the methods used, discussing the development of new techniques where relevant.

2.7.1 Principles of metabolic tracer techniques

Atoms consist of a tightly packed central core of positively charged and neutral subatomic particles known as protons and neutrons respectively, the number of which determines its mass (denoted by the symbol A) in atomic mass units (AMUs). The number of protons is given by the symbol Z such that the fictitious molecule X can be written as follows:

$${}^{A}_{Z}X$$

Otherwise identical atoms can differ in mass due to variations in the numbers of neutrons, thereby altering the mass number. These atoms are otherwise chemically

identical and are known as *isotopes*. Both ^{12}C and ^{13}C are examples of stable isotopes which, unlike radioactive isotopes e.g. ^{14}C, do not spontaneously decay to produce radiation (and other elements). Stable isotopes are naturally occurring, and can be measured using gas chromatography mass spectrometry, with ^{13}C contributing to ~1.1% of the total carbon pool, whereas radioisotopes are measured by scintigraphy. Both radioactive and stable isotopes can be used to label molecules for use in metabolic tracer studies e.g. in Chapter 4 I used 1,2,3,4-[$^{13}C]_4$-palmitate to study fatty acid metabolism.

A *tracer* can be defined as a compound which is chemically and functionally identical to the naturally occurring *tracee* compound of interest but is distinct in some way that enables its detection. The detectable nature of the tracer then allows information to be gathered on the metabolism of the tracee compound in the body (Wolfe 1992).

The use of stable isotopes as metabolic tracers began in the 1930s with the use of ^{2}H, ^{13}C and ^{18}O (Dole M 1935;Schoenheimer and Rittenberg 1935;Wood et al. 1941), and actually preceded the use of more easily detectable radioactive tracers which became more popular following their increase in availability after World War II. The use of stable isotopes then had a resurgence in the 1970s in line with an improved sensitivity of gas chromatography mass spectrometry (Wolfe 1992). Stable isotopes also pose little or no risk to human subjects as they contain no radioactivity and can be used repeatedly in a single volunteer.

There are three main ways in which tracers are used in metabolic research, the latter two were employed in the experiments undertaken in Chapter 4.

i) to measure a compound's kinetics following bolus administration
ii) to measure the tracer's incorporation into another compound to calculate a rate of synthesis e.g. of hepatic *de novo* lipogenesis

iii) to determine the rate of substrate turnover or its rate of appearance into the plasma using the tracer dilution technique e.g. to quantify the rate of appearance of fatty acids in the plasma.

2.7.2 Isotope dilution technique terminology

2.7.2.1 Rate of appearance

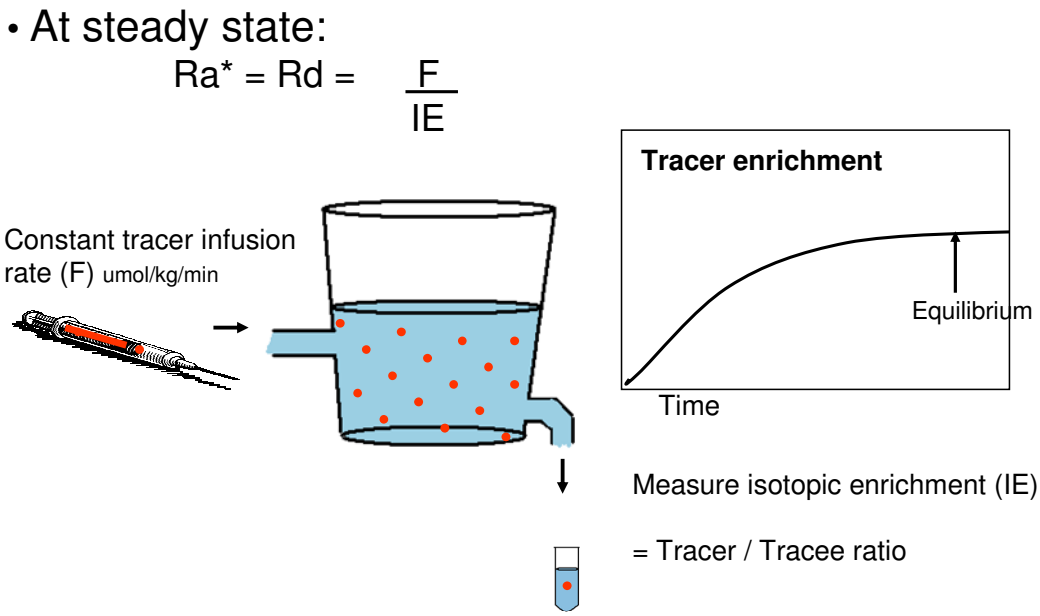
Rate of appearance (R_a) is a key term in metabolic tracer studies employing the isotope dilution technique (see Figure 2.1). To understand the isotope dilution technique it is necessary to conceptualise a substrate pool within the body (represented by the bucket of water in Figure 2.1) with the assumption that the pool is homogenous and freely mixing. At steady state, the influx (or R_a) of water is equal to output (or rate of disappearance [R_d]). If we then infuse a tracer at a constant rate for long enough for it to reach equilibrium then $R_a = R_d$, and at this time point of isotopic equilibrium the ratio of tracer to tracee will remain constant at a plateau. R_a of substrate can then be calculated using the formula (Steele 1959):

R_a *(μmol/kg per min) = F/IE,*

where F is the isotope infusion rate (μmol/kg per min) and IE is the isotopic enrichment at plateau expressed as the tracer to tracee ratio (TTR)

Figure 2.1 Principles of the isotope dilution methodology

Once steady steady of tracer enrichment has been achieved, the rate of appearance (R_a) of the tracee in plasma is calculated using the equation as shown where F is the tracer infusion rate and IE is the isotopic enrichment or tracer to tracee ratio.



The R_a of a compound is also referred to as the turnover rate or occasionally the flux rate.

It is noteworthy that the R_a is not dependent on the actual amount/duration of tracer infused, only that a steady state is achieved. The time to achieve steady state is dependent on the turnover rate of the substrate (and the pool size), with long infusion periods needed for substrates with a slow turnover rate. A priming dose of tracer is therefore often used in this situation with the aim of achieving isotopic equilibrium more rapidly, without altering the final enrichment plateau.

The required priming dose (P, umol/kg) can be expressed as a function of the pool size and the substrates R_a (Wolfe 1992) using the equation:

$P/F = C*V/ R_a$

Where *C* is the concentration of the unlabelled trace in µmol/ml and *V* is the volume of distribution (ml/kg). Therefore if there is some knowledge of the pool size and expected R_a then the P/F ratio can be estimated and pilot studies used to determine if the chosen values are satisfactory. F represents the tracer infusion rate as before.

The isotope dilution technique was used in Chapter 4 using a labelled palmitate tracer to measure the R_a of the fatty acid palmitate in plasma, representing the release of fatty acids from stored triglycerides into the circulating free fatty acid pool in mice. It follows that in addition to knowing the tracer infusion rate the istopic enrichment must be measured as will be described below.

2.7.2.2 Isotopic enrichment

When a radioactive tracer is used isotopic enrichment is expressed as specific activity (SA) which represents the ratio of the amount of measured radioactive tracer/measured amount of unlabelled tracee (Wolfe 1992). However, when a stable isotopic tracer is used its concentration cannot be measured directly. Additionally, "background" amounts of stable isotopes create indistinguishable naturally occurring mass isotopomers which need to be subtracted from the measured enrichment during the study. The unit tracer/tracee ratio (TTR) is therefore used as a measure of enrichment and represents the molar ratio of two discretely measurable species. In practice, what is measured is isotope abundance ratios obtained using GCMS. However, these are not identical to the true TTR due to overlap in spectra between the tracer and tracee, and possible other artefacts produced by GCMS. Corrections therefore have to be made to determine the true TTR, which can be achieved mathematically (using skew correction factors to correct for overlapping spectra) or alternatively a standard curve can be used which also takes into account some GCMS artefacts (Patterson et al. 1998).

A series of standard solutions of known isotopic enrichment are used to construct a standard curve such that the measured TTR determined by GCMS can be corrected to the "true TTR".

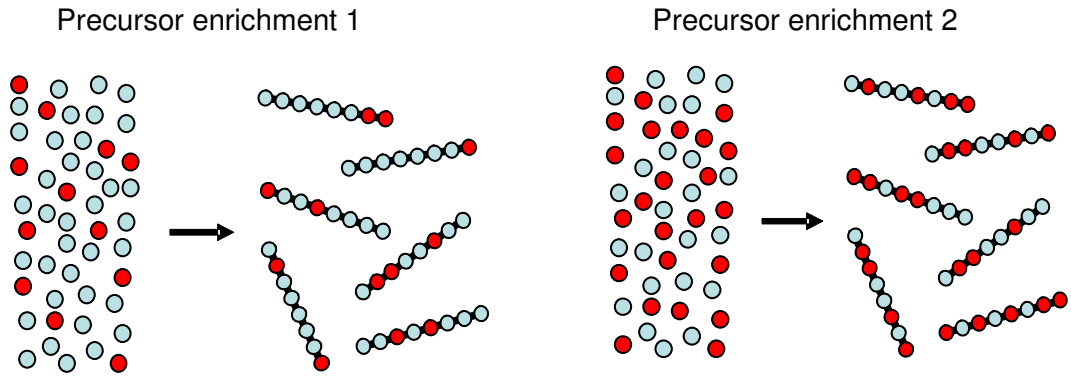
2.7.2.3 Mass isotopomers distribution analysis (MIDA)

The term *isotopomer* is used to describe a molecule into which a tracer has been incorporated. Positional isotopomers differ in respect to the site of incorporation of the isotopic tracer, whereas mass isotopomers differ by mass.

MIDA is a technique used to enable calculation of the rate of synthesis of a polymer synthesised from a monomer (Chinkes et al. 1996). When the monomer is labelled then its incorporation into the polymer will lead to the production of mass isotopomers of the polymer product. Measuring the relative abundance of the different mass isotopomers by GCMS makes it possible to indirectly calculate the enrichment of a labelled monomer precursor pool necessary for the calculation of synthetic rates using the precursor product relationship as described below. Figure 2.2 demonstrates how different precursor pool enrichment leads to different distributions of mass isotopomers.

Figure 2.2 Comparison of precursor pool enrichments for MIDA

Red circles represent the labelled precursor molecule to be incorporated into the synthesised polymer. The mass isotopomer distribution in the product is dependent on the degree of precursor enrichment. Analysis of the pattern of mass isotopomers distribution, determined using gas chromatography/mass spectroscopy allows calculation of the enrichment of the precursor pool.



The MIDA approach was developed simultaneously by Hellerstein's (Hellerstein and Neese 1992) and Kelleher's (Kelleher and Masterson 1992) groups, with slight variations adopted by others (Chinkes et al. 1996;Lee et al. 1992). All are based on the same underlying principle of a combinatorial precursor-product biosynthesis model correcting for naturally occurring isotopomers which interact with a varyingly diluted enriched precursor pool (Chinkes et al. 1996). Comparison of these slightly varying methodologies have shown the results to be very similar (Chinkes et al. 1996). However, because the models and algorithms of Hellerstein and Kelleher do not use conventional expressions of isotopic enrichment (TTR or MPE), and because they require the use of graphical matrices and "look up" tables to calculate the precursor enrichment, I opted to use the method of Chinkes *et al* (Chinkes et al. 1996) which uses algebraic equations and calculates palmitate enrichment using TTRs.

2.7.2.4 Calculation of precursor enrichment using the method of Chinkes *et al*

To understand this method let us consider the synthesis of palmitate from eight acetyl CoA subunits using labelled 1-$^{13}C_1$-acetate. Prior to administration of any tracer

naturally occurring mass isotopomers exist with the theoretical relative abundance 100%, 19.12%, and 2.14% for methyl palmitate M+0, M+1, and M+2 respectively (Chinkes et al. 1996). It should be noted that due to hydrogen abstraction and GCMS artefacts the actual measured isotope abundance ratios may significantly differ from these theoretical values (Patterson et al. 1998) and the measured background values should therefore be used. Any additional incorporation of 1-$^{13}C_1$-acetate into palmitate will alter this distribution of mass isotopomers dependent on the enrichment of labelled acetate.

From Figure 2.2 we can see that if 50% of the acetyl CoA precursor pool is labelled then on average each palmitate molecule will contain four labels corresponding to M+4 measured by GCMS. In comparison, if only 25% of the precursor pool is labelled on average each palmitate molecule will contain two labels, demonstrating that different precursor enrichments produce characteristic mass isotopomer patterns or distributions. In reality the palmitate pool will be diluted by pre-existing unlabelled palmitate. However, the relative distribution of the (newly synthesised) mass isotopomers will remain the same and so using the MIDA technique the precursor enrichment can still be calculated.

The method requires the assumption that the precursor pool enrichment (not necessarily the product enrichment) is constant over time, and requires that a minimum of three peaks are measured.

Firstly, TTRs are calculated for the M+1 and M+2 isotopomers using the following formulae.

$$TTR[M+1] = (M+1/M+0)_{post} - (M+1/M+0)_{pre}$$

$$TTR[M+2] = (M+2/M+0)_{post} - (M+2/M+0)_{pre} - dT_1 * TTR[M+1]$$

Where dT1 is the theoretical percentage of singly labelled product that has a heavy atom in a position other than the labelled position. This removes the contribution of singly labelled (M+1) palmitate to the M+2 peak due to overlapping spectra.

Assuming precursor enrichment (*p*), is at steady state it can be calculated from the TTRs, and expressed in moles percent excess (MPE), as follows:

$$p = (2 * TTR[M+2]/TTR[M+1])/((n-1) + 2 * (TTR[M+2]/TTR[M+1]))$$

where *n* is the number of precursor molecules used to synthesis one product molecule e.g. 8 acetate molecules are contained in one palmitate molecule.

Precursor and product enrichment must be expressed in the same units. Therefore the precursor enrichment can be converted from units of MPE precursor enrichment to MPE of singly labelled product. In the case of an acetate tracer, acetyl CoA enrichment is converted to palmitate enrichment. The formula used to calculate singly labelled product enrichment (EF) is:

$$EF = n*p*(1-p)^{n-1}$$

The equation corrects for the probability that a randomly formed product has one label incorporated, using *p* the calculated percentage of labelled precursor molecules i.e. the precursor (acetyl CoA) enrichment in MPE.

The fractional synthesis rate i.e. the fraction of the tissue TG-palmitate pool that is newly synthesised, per unit of time, can now be calculated using the formula:

$$FSR = [E_b(t2) - E_b(t1)]/[(t2-t1)*EF]$$

Where E_b is the product enrichment at timepoints t2 and t1 and EF is the precursor enrichment (converted to singly labelled product enrichment).

The absolute synthesis rate (ASR) is the calculated amount of product that has been synthesised over a specific time period.

ASR = FSR * pool size

Thus, when comparing groups it is possible for the FSR to be lower in one group but its ASR to be higher than a comparison group if its pool size is greater.

In Chapter 4 we opted to use doubly labelled $[^{13}C]_2$-acetate as recommended by Chinkes *et al* to improve the specificity of the analysis (Chinkes et al. 1996). The method is essentially unchanged except M+2 and M+4 are used instead of M+1 and M+2, thereby reducing the background enrichment to be subtracted. dT2, used to correct for overlapping spectra, is 1.75%.

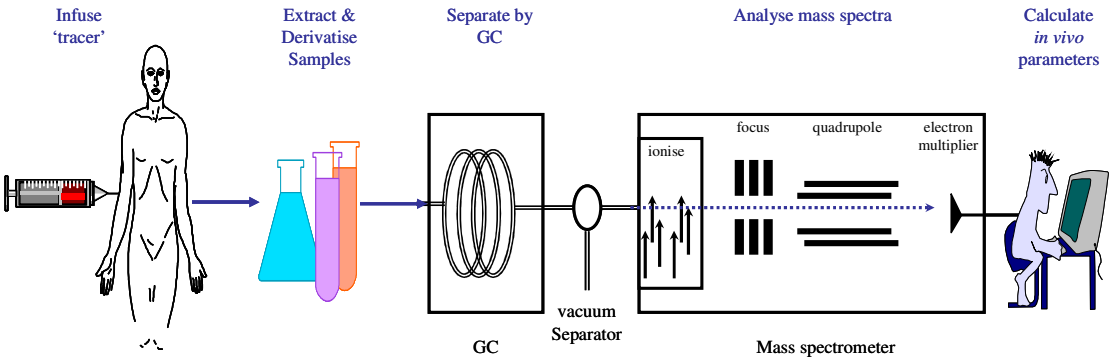
2.8 BASICS OF GAS CHROMATOGRAPHY/ MASS SPECTROMETRY (GCMS)

Gas chromatography provides a physical method for separating and quantifying components of complex mixtures of organic compounds and was therefore used in Chapter 4, in combination with mass spectrometry, to measure isotopic enrichment in the metabolic tracer studies. Separation of compounds is achieved by the partitioning of different components into two phases; stationary and mobile. In gas chromatography the stationary phase is a liquid or polymer film bonded to the inside of a capillary tube (or *column*), and the mobile phase is a pressurised carrier gas e.g. helium, that moves through the capillary tube. Components that spend more time in the gas/mobile phase therefore move along the column more rapidly. Factors influencing time spent in the mobile phase include temperature, and the chemistry of the stationary phase which effects the interaction with the components of interest. The *retention time* describes the time taken to pass through the column is represented on the gas chromatogram by a corresponding peak which gives some indication on the nature of the molecule (Goodman 1980).

Gas chromatography is a very sensitive tool for separating complex mixtures, but on its own provides only limited information on the identity of eluted molecules. It requires to be coupled with a mass spectrometer to provide more detailed information by measuring molecular weight. The output of the mass spectrometer is the mass spectrum and aids identification of molecules based on their mass and pattern of breakdown or fragmentation within the instrument following ionisation. Ions are presented as a bar graph by their relative abundance and mass to charge ratio (*m/z* ratio), where *m* is the mass in Daltons (Da) and *z* is the charge of one electron, which usually equals 1. A diagram of a GCMS setup is shown in Figure 2.3.

Figure 2.3 Sample analysis and instrumentation by gas chromatography mass spectrometry

Schematic diagram demonstrating the necessary steps required to analyse stable isotopes infused during a metabolic tracer study by gas chromatography/mass spectrometry (GCMS).



Blood samples are taken during the stable isotope infusion. The compound(s) of interest can then be extracted from plasma for analysis by GCMS. Derivatisation describes the process whereby components of interest are chemically altered to make them more volatile and suitable for separation by gas chromatography e.g. fatty acids are derivatised to fatty acid methyl esters (FAMEs) (Patterson et al. 1999).

A small volume of the derivatised sample is then injected onto the analytical column through the inlet system which vapourises the sample. The column is also maintained

at temperature and is housed within an oven. The GC operates at atmospheric pressure, whereas the MS operates within a vacuum to prevent interference form particles with air.

The mass spectrometer contains three main components: the ion source; the analyser; and the detector. The capillary column is coupled, through a heated sheath, to the ion source which is kept under vacuum. Molecular ionisation is required to allow mass measurement, and two methods can be employed within the ion source; *electron impact ionisation* (EI) and *chemical ionisation* (CI). In both methods, the sample passes through a beam of electrons, although in CI, a reagent gas (typically methane) is present in the ion source which indirectly ionises the analyte. The higher energy EI method is used more commonly but leads to greater fragmentation of the molecular ions. Once molecular ions are produced they pass through focusing lenses into the analyser component of the mass spectrometer. A number of different types of analysers exist, but commonly either magnetic or quadrupole mass analysers are used. Ions are separated depending on their mass to charge ratios which are then collected by the detector plate the signal converted to allow data analysis by computer. Typically a mass spectrum is scanned every 0.2 to 0.5 seconds.

Total ion current or total ion chromatogram (TIC) represents the sum of all mass spectra produced by the GCMS system, and each peak is therefore a sum of all the fragments/isotopomers of that molecule. TIC mode is often used to initially identify the time at which a standard is eluted from the column.

Selective ion monitoring (SIM) is an alternative to TIC and allows the mass spectrometer to measure only specific ions of interest, increasing sensitivity. SIM mode is commonly employed in metabolic tracer studies to measure the mass isotopomers corresponding to the tracer and tracee molecule for calculation of the TTR. Knowledge of the tracer's molecular fragmentation pattern can therefore be important to ensure monitoring of the fragment containing the tracer atom is undertaken.

2.9 ASSAY DEVELOPMENT

The isotope dilution technique and mass isotopomers distribution analysis methods employed in Chapter 4 required GCMS analysis of fatty acids. GCMS assay development was therefore undertaken for fatty acids, as analysis of these tracers had not previously been performed locally.

2.9.1 GCMS analysis of palmitic acid

Palmitic acid is a 16 carbon (C16:0), saturated fatty acid constituting approximately 30% of plasma free fatty acids. Isotopes of palmitate (e.g. ^{14}C-palmitate, U-^{13}C-palmitate, D2-palmitate) infused in albumin are used to study fatty acid kinetics and are representative of plasma fatty acids (Mittendorfer et al. 2003). After initially developing the assay for a singly labelled $^{13}C_1$-palmitate tracer, commonly used in human studies, we opted to use a 1,2,3,4-[^{13}C]$_4$-palmitate to improve specificity. Stable isotopes of palmitate are safe, and combined with GCMS provide a relatively sensitive and accurate means of undertaking metabolic tracer studies avoiding the exposure to radioactivity.

Prior to GCMS analysis FFAs must be derivatised to fatty acid methyl esters (FAMEs), but first must be extracted from plasma using a variety of solvents as water inhibits the generation of FAMEs (Patterson et al. 1999). FFAs have classically been separated from total extracted lipids by thin layer chromatography, and FAMEs prepared using derivatising agents such as boron trifluoride and methanol (Morrison and Smith 1964;Wolfe 1992). However, this is time consuming, can lead to breakdown of FFAs (chemical fractionisation), reducing recovery of some fatty acid species, and is less efficient than more modern methods (Patterson et al. 1999). We therefore elected to use the method of Patterson *et al* (Patterson et al. 1999) derivatising FFAs using iodomethane (dissolved in dichloromethane) which importantly has not been shown to react with or hydrolyse triglycerides or other esterified lipid species to produce interfering FAMEs (Patterson et al. 1999).

GC analysis was initially undertaken using a Hewlett Packard 5890 series II gas chromatograph (Palo Alto, Ca) with a BPX5 25m x 35mm id 0.25µm film Forte GC capillary column (SGE, Milton Keynes, UK) interfaced to a mass spectrometer (ThermoQuest Trio-1) operated in electron impact ionisation mode (70eV) using source and interface temperatures of 200ºC and 250ºC respectively.

.

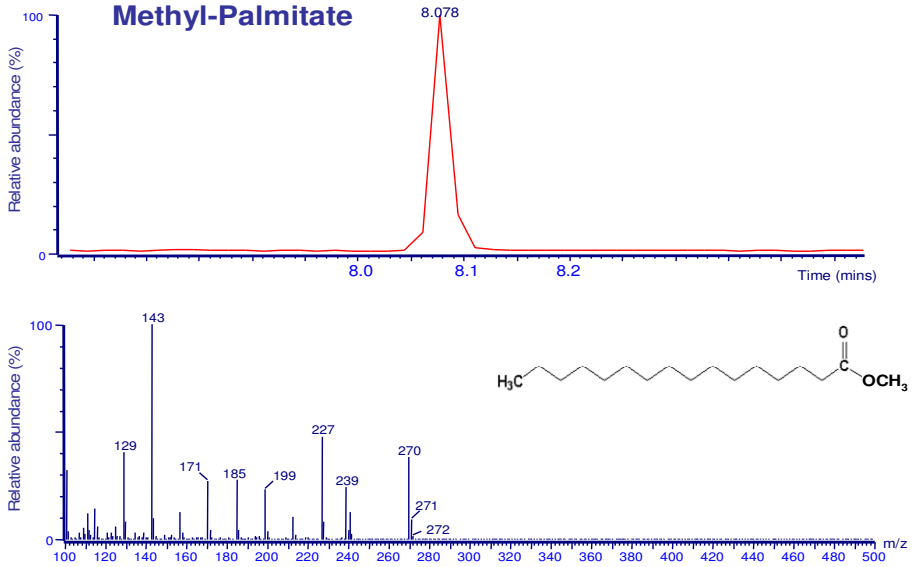
To optimise GCMS conditions, stock solutions of unlabelled and $^{13}C_1$- palmitate (1µg/µl in chloroform) were prepared and 250µl aliquots reduced to dryness under oxygen free nitrogen at 60^{o}C in glass Reactivials. As per the final stages of the Patterson method (Patterson et al. 1999) TBHAS buffer (250µl) and iodomethane derivatising solution (250µl) were added and samples vortexed (10mins) to form FAMEs. FAMEs were then extracted into hexane (3ml), reduced to dryness under oxygen free nitrogen at 60^{o}C, and redissolved in heptane (150µl) for analysis by GCMS.

Palmitate and $^{13}C_1$- palmitate solutions were first analysed in *total ion chromatogram* (TIC) mode and the appropriate mass/charge (m/z) ratios of the FAMEs derivatives identified (See Figure 2.4A). Subsequently *selected ion monitoring* (SIM) was used to measure ^{12}C methyl palmitate as molecular ion $[M+0]^+$ at m/z 270 [retention time (t_R) 8.1min], ^{13}C methyl palmitate as $[M+1]^+$ at m/z 271 (Figure 2.4B). A series of injection temperatures were assessed from 200-300^{o}C and areas under the curve compared for peaks identifying 280^{o}C as the optimum. A splitless injection system was used and 1µl of sample injected, with a 5 second pull out delay. The GC was programmed from an initial 1minute hold at 60^{o}C, and then the temperature was increased to 220^{o}C with a 25^{o}C/minute ramp, then to 300^{o}C with a 10^{o}C/minute ramp with a 1minute final hold.

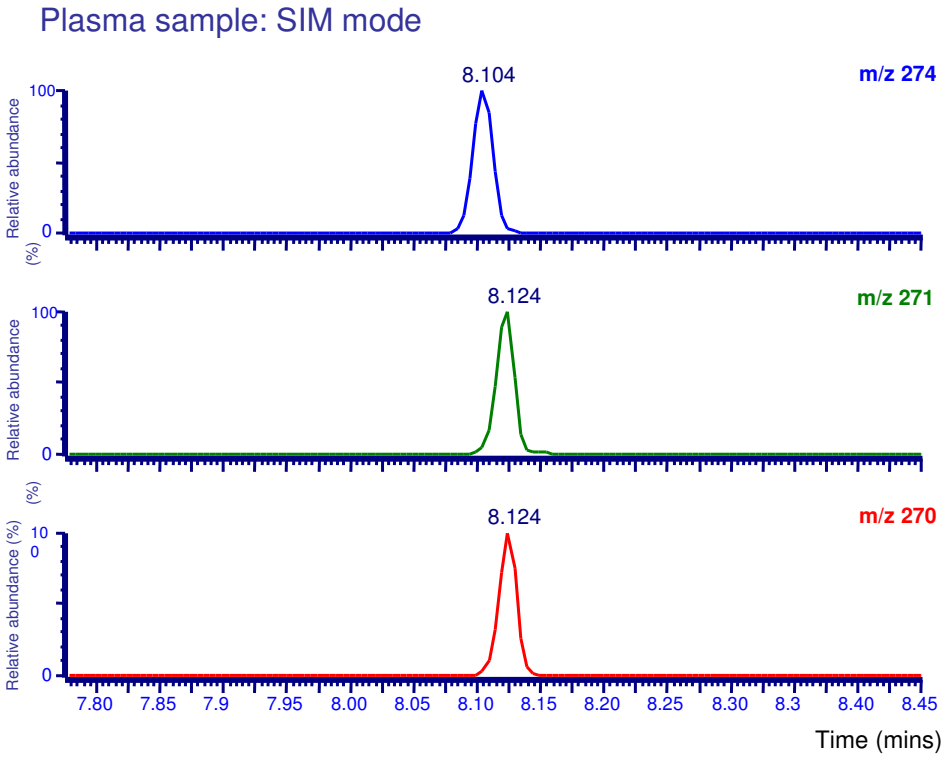
Figure 2.4 GCMS chromatograms for methyl palmitate isotopomers

A) Mass chromatogram and corresponding mass spectrum for methyl palmitate, B) Selective ion monitoring mode for D4 methyl palmitate internal standard (m/z 274), C13 methyl palmitate (m/z 271), and methyl palmitate (m/z 270) recovered from human plasma.

A)



B)

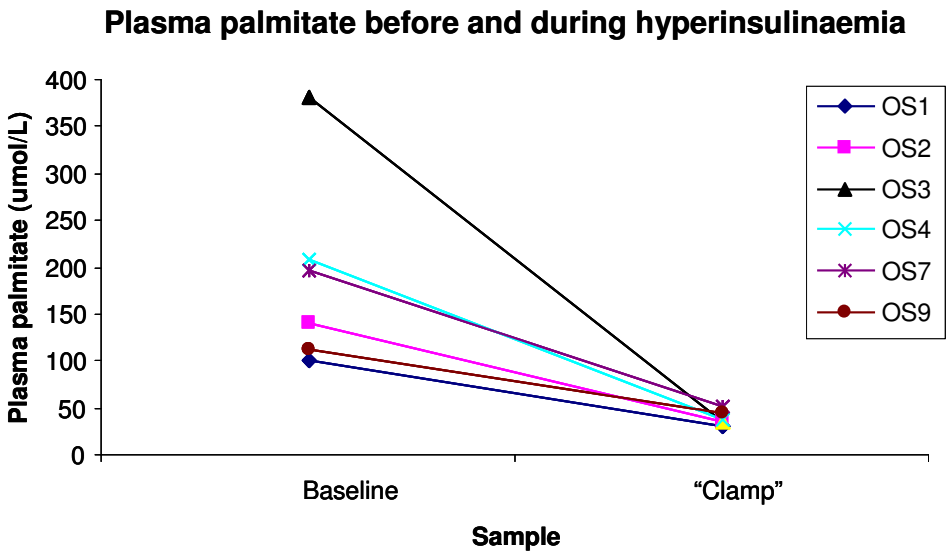


Subsequent steps of the Patterson method (Patterson et al. 1999) for the extraction of plasma FFAs and derivatisation to FAMEs were added, using aliquots of the stock solutions with the addition of an internal standard (initially D4-palmitate, although this was later changed to heptadecanoic acid with the 1,2,3,4-[$^{13}C]_4$-palmitate tracer) to improve reproducibility. Ultimately the full assay was successful in extracting palmitate from plasma (250µl) with addition of internal standard (10µg).

Xcalibur software (ThermoFinnigan, Austin, Tx, USA) was used to calculate areas under the curve and integrate peaks corresponding to labelled and unlabelled palmitate, as well the internal standard. Palmitate standard curves were prepared using samples of known quantities/concentrations of palmitate and were deemed acceptable if R values were >0.99. Plasma samples could then be analysed to measure palmitate concentrations, and the reproducibility of the technique assessed, producing a coefficient of variability of precision (n=5) of less than 2%. To assess the ability of the assay to measure dynamic changes in plasma concentrations, analysis of stored plasma samples from a previous study was undertaken (Sandeep et al. 2005). Samples were analysed from baseline and during hyperinsulinaemic clamp (expected to suppress plasma FFAs) (Figure 2.5).

Figure 2.5 Dynamic changes in plasma palmitate concentrations

Palmitate concentrations before and during hyperinsulinaemia (OS; obese subject code).



Given that mice generally have higher levels of FFAs than humans, and because only small volumes of plasma would be available from mice, the method was scaled down to use 50µl of plasma. An alternative, more sensitive, GC-MS instrumentation was also used as described in Section 2.9.3.

Murine plasma free fatty acids were extracted and derivatised using a modified method of Patterson *et al* (Patterson et al. 1999) as follows. Aliquots of plasma (50µl), water (150µl), and internal standard (5µg), plus heptane (140µl) were added to 13 x 10mm screw top *Pyrex* glass reaction tubes. Samples were vortexed (3mins) prior to addition of ice cold acetone (3ml) to precipitate plasma proteins, briefly vortexed again, and placed at -20^{o}C for 15 minutes. Samples were then centrifuged (1500 x g, 5mins, 4^{o}C) and the supernatant transferred to fresh glass reaction tubes. Hexane (2ml) and water (2ml) were added before mixing gently on a horizontal platform shaker (15mins, room temperature) and samples centrifuged again as before. The upper lipid containing solvent phase was then transferred fresh glass reaction tubes and reduced to dryness under oxygen free nitrogen at 60^{o}C. TBHAS buffer (250µl) and iodomethane derivatising solution (250µl) were added and samples vortexed (10mins) to form FAMEs. FAMEs were extracted into hexane (3ml), reduced to dryness under oxygen free nitrogen at 60^{o}C, and redissolved in heptane (150µl) for analysis by GCMS.

2.9.2 Tissue and plasma TG-palmitate enrichment

To measure the uptake/flux of fatty acids into plasma and tissue TG pools it is first necessary to isolate the TG lipid component, prior to hydrolysis of the TG ester bonds, liberating constituent fatty acids (including the palmitate tracer) for derivatisation to FAMEs. Again these techniques had not previously been undertaken in our lab and a modification of the classic Folch (Folch et al. 1957) extraction method was used to extract plasma and tissue lipids. This was combined with solid phase extraction (SPE) to isolate the TG lipid fraction (Burdge et al. 2000). SPE was chosen in preference to thin layer chromatography for separation of lipid classes as it

is less time consuming, and has been shown to obtain superior results, with no cross contamination of lipid classes, and good recovery (Burdge et al. 2000). Polar and neutral lipids (phospholipids and FFAs, and cholesterol esters [CEs] and TGs respectively) were separated by SPE, followed by further separation using solvents of increasing polarity as described below.

The published method (Burdge et al. 2000) also describes elution of FFAs using chloroform-methanol-acetic acid (100:2:2, v/v) following prior elution of phospholipids by chloroform methanol (3:2, v/v) and methanol washes. This proved to be unsuccessful due to marked contamination seen in blanks and lack of sensitivity with the small volume of plasma analysed. This "contamination" was present when blanks derivatised by both iodomethane and sulphuric acid methanol suggesting that it was not contamination due to TGs. Possible plasticisers present within the column and manifold may have been to blame and contamination appeared to be reduced when the manifold was not used to drain the columns.

To measure plasma TG-palmitate enrichment plasma lipids were extracted from 50µl of murine plasma using triheptadecanoin (5µg in chloroform) as an internal standard using a modified Folch technique (Folch et al. 1957). Plasma and internal standard were added to 13 x 10mm screw top *Pyrex* glass reaction tubes, followed by chloroform-methanol (2ml) solution, and samples mixed for 15 minutes at room temperature. 1M NaCl (300µl) solution was added and samples briefly vortexed prior to centrifugation (1125 x g at 4°C for 10mins). The aqueous phase was removed and the lower organic phase aspirated and transferred to a new reaction tube. The interfacial protein disc was vortexed for 15minutes in chloroform-methanol solution (1ml) and 1M NaCl (150µl), followed by centrifugation as above. The organic phase was aspirated and combined with the first extract, then reduced to dryness under oxygen free nitrogen at 60°C, prior to being redissolved in chloroform (1ml) for solid phase extraction (SPE) to separate TGs as described below (Burdge et al. 2000).

A similar method was used to extract TGs from liver tissue. Tissue (200mg) was homogenised in 40 volume of chloroform:methanol solution and sonicated for

15mins (Ametaj et al. 2003). 1M NaCl solution (0.2 volume) was added, and the samples briefly vortexed, prior to centrifugation (2000 x g for 20minutes at 10^{o}C). The lower organic phase was extracted and the upper methanol and NaCl containing phase discarded. The remaining homogenate was redissolved in 1ml chloroform and filtered. Following additional chloroform washes (1ml and 2ml), the chloroform was added to the original organic phase and the mixture reduced to dryness under oxygen free nitrogen at 60^{o}C. The samples were then redissolved in chloroform (1ml) for SPE.

2.9.2.1 Triglyceride separation by solid phase extraction

Aminopropyl silica cartridges (Bondelut Varian, 100mg packed silica per 1ml column, Speck and Burke analytical, UK) were placed in a vacuum manifold and preconditioned with chloroform (2mls). Glass collection tubes were placed and samples applied under gravity, removing the residual with vacuum. Two further chloroform (1ml) washes were applied, and this first fraction containing cholesterol esters and TGs reduced to dryness under oxygen free nitrogen at 60^{o}C. The eluted neutral lipids were redissolved in hexane (1ml) and a further set of aminopropyl silica cartridges preconditioned with hexane (4 x 1ml). The sample was applied under gravity, followed by hexane washes (2 x 1ml) under vacuum to elute cholesterol esters. Collection tubes were placed and TGs eluted under vacuum with hexane-chloroform-ethylacetate (100:5:5, v/v) (2 x 1ml). The void fraction was reduced to dryness under oxygen free nitrogen at 60^{o}C.

2.9.2.2 Triglyceride hydrolysis and derivatisation

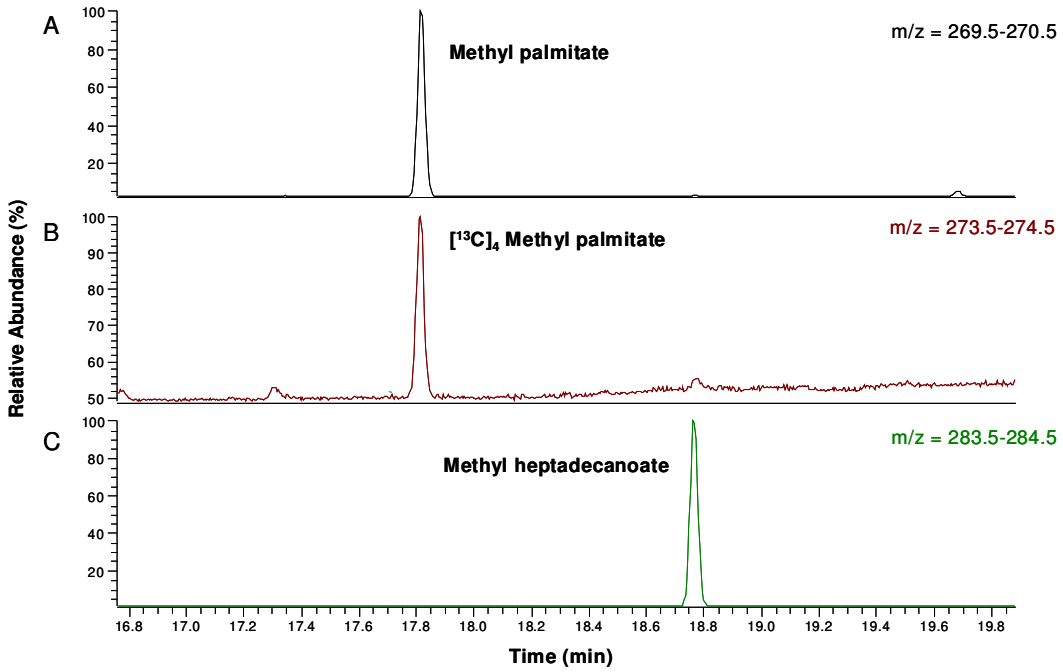
The eluted TGs were redissolved in toluene (1ml), briefly vortexed and derivatised using acidified methanolic sulphuric acid (2% v/v), incubating for 18 hours at 50^{o}C (Burdge et al. 2000). The reaction mixture was then neutralised with buffer ($KHCO_3$ [0.25M] and K_2CO_3 [0.5M]). The lipids were extracted into hexane (2ml) by mixing for 15 minutes on a horizontal platform shaker and centrifuging (1125 x g for 10mins at 14°C). The hexane layer was aspirated and reduced to dryness under oxygen free nitrogen at 60^{o}C and samples redissolved in heptane (75µl for plasma samples and 200µl, followed by 1 in 200 dilution for tissue samples) for analysis by GCMS.

2.9.3 Gas chromatography mass spectrometry instrumentation

Electron impact ionization GC-MS analysis was undertaken on a ThermoFinnigan *Voyager* gas chromatograph mass spectrometer with an Agilent HP-Innowax column (30m, 0.320mm, 0.5µm) operated in electron impact ionization mode (70eV). Source, interface and injection temperatures were 200°C, 250°C and 260°C respectively. The column temperature was programmed from a 1.5minute hold at 50°C, with a 13°C/min ramp and 1.5minute hold at 260°C. Fatty acid methyl esters derived from plasma FFA and TG pools were analysed using selective ion monitoring of molecular ions with *m/z* 270, 272, 274 and 284, corresponding to [M+0], [M+2], and [M+4] isotopomers of methyl palmitate and the methyl heptadecanoate internal standard respectively (see Figure 2.6). To avoid concentration dependent effects of methyl palmitate on ionization, each sample was analysed several times using a range of injection volumes as previously described (Patterson et al. 1998), and tracer to tracee ratios determined from a standard curve.

Figure 2.6 GCMS chromatogram measuring endogenous and exogenous fatty acid methyl esters depicting m/z ratios

Chromatograms depicting m/z rations measuring A) Methyl palmitate, B) [$^{13}C]_4$ methyl palmitate, and C) methyl heptadecanoate.



2.10 OTHER EX VIVO ASSAYS

2.10.1 Free fatty acids

Plasma FFAs were analysed in duplicate using a commercial colourimetric kit (Wako, Fuggerstrabe, Germany) utilising acyl CoA synthase (ACS) and acyl coA oxidase (ACOD).

In the presence of adenosine triphosphate (ATP), magnesium ions and co-enzyme A (CoA), ACS converts FFAs to the thiol esters of CoA, acyl-coA, as well as adenosine monophosphate (AMP) and pyrophosphate (PPi). Acyl-coA is then oxidised by ACOD to produce hydrogen peroxide, which in the presence of peroxidase (POD) and two reagents (4-amino antipyrine and methyl-N-ethyl-N-(betahydroxyethyl) aniline [MEHA]) yields a purple coloured product.

A standard curve was prepared from a 1000μmol/l FFA (oleic acid) standard by serial dilutions with the provided buffer solution, including a buffer blank. Plasma (and standard curve samples) were incubated at 37°C for 10mins with each of the two reagents ("A" and "B"), then allowed to equilibrate to room temperature. After ensuring no bubbles were present, absorbance was measured at 540nm using a platereader. The standard curve was deemed acceptable if $R^2 > 0.98$, and samples/standards run in duplicate, accepting a variance of <10%. The FFA content of each plasma sample was calculated and corrected for sample volume to μmol/l.

2.10.2 Plasma triglycerides

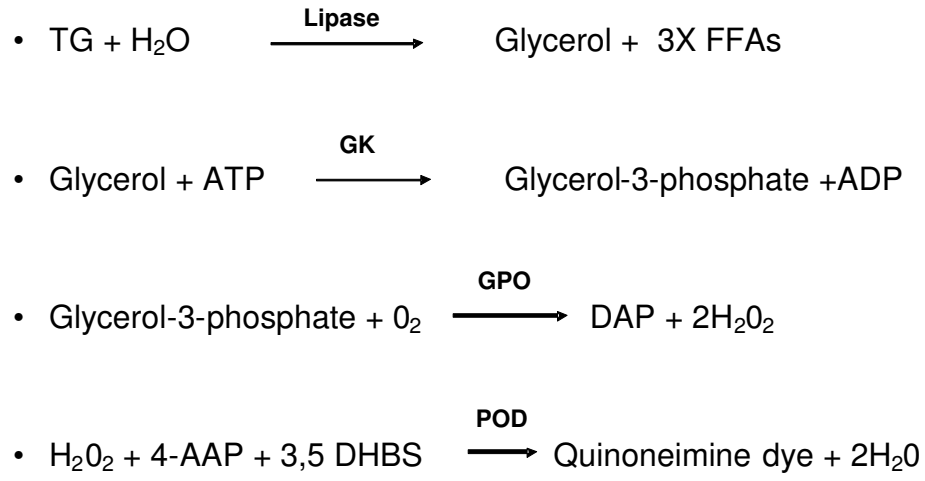
TGs were measured enzymatically with the Infinity triglycerides liquid stable reagent (ThermoTrace, Noble Park, Australia).

Briefly, TGs were first hydrolysed to FFAs and glycerol by a lipase and subsequently phosphorylated to glycerol-3-phosphate by glycerol kinase (GK), consuming ATP. Hydrogen peroxide (H_20_2) was then produced by the oxidation of glycerol-3-

phosphate to dihydroxyacetone phosphate (DAP) by glycerolphosphate oxidase. A peroxidase enzyme (POD) then reacts H_20_2 with two reagents (4-amino antipyrine and 3,5-dichloro-2-hydroxybenzene sulphate to produce a red coloured dye (see Figure 2.8). The absorbance of the dye was proportional to the concentration of TG in the sample.

Figure 2.8 Triglyceride assay reaction

Summary of the steps involved in a commercial triglyceride assay.



200µl of the reagent mix was added to 2µl of sample. A calibration standard (2µl, 2.5mmol/l) and blank were also measured. The mixture was incubated at 37°C for 5minutes and a platereader used to measure absorbance at 500nm. Samples were analysed in duplicate when possible, accepting a variance of 10%.

Plasma TGs (mmol/l) = [Average OD of sample/ average OD of standard] x 2.5

2.10.3 Liver triglyceride concentration

To measure hepatic TG content, 100mg of liver was homogenised in 20 volumes of propan-2-ol. The homogenate was shaken in an orbital shaker for 45 minutes, centrifuged at 3000 x g for 10 minutes at 4ºC and 2µl of the supernatant assayed using a commercial triglyceride kit (ThermoTrace) as described above. Results were converted to µmol/g liver and µmol TG/liver, the latter representing the total liver TG pool.

2.10.4 Bilirubin

Plasma bilirubin concentration was determined using a commercial kit (Alpha laboratories Ltd, Eastleigh, UK) adapted for use on a Cobras centrifugal analyser (Roche Dignostics Ltd, Welwyn Garden City, UK). The assay is based on an acid diazo method (Pearlman and Lee 1974). Both conjugated and unconjugated bilirubin react with diazotised sulphanilic acid to produce an azobilirubin, the absorbance of which is proportional to that of bilirubin in the sample measured at 550nm. Standards were run in duplicate, with a variance of <10% and an R value greater than 0.99 deemed acceptable.

2.10.5 Alanine aminotransferase

ALT activity was measured using a commercial kit (Randox laboratories, Co. Antrim, UK) based on the method of Bergmeyer (Bergmeyer et al. 1978), adapted for use on a Cobras centrifugal analyser (Roche Dignostics Ltd, Welwyn Garden City, UK). In summary, ALT converts L-alanine and 2-oxoglutarate to pyruvate and L-glutamate. In the presence of lactate dehydrogenase and NADH the pyruvate is reduced to lactate, whilst the oxidation of NADH to NAD^+ produces a chromphore, the abundance of which may be quantified at 340nm. Standards were run in duplicate, with a variance of <10% and an R value greater than 0.99 deemed acceptable.

2.10.6 Aspartate aminotransferase

AST activity was measured using a commercial kit (Randox laboratories, Co. Antrim, UK) based on the method of Bergmeyer (Bergmeyer et al. 1978), adapted for use on a Cobras centrifugal analyser (Roche Dignostics Ltd, Welwyn Garden City, UK). ALT converts oxoglutarate and L-aspartate to form L-glutamate and oxaloacetate. In the presence of NAPH and malate dehydrogenase, oxaloacetate is converted to malate and NAD^+ leading to a chromophore, the abundance of which may be quantified at 340nm. Standards were run in duplicate, with a variance of <10% and an R value greater than 0.99 deemed acceptable.

2.10.7 Plasma leptin

Plasma leptin levels were measured using an ultrasensitive mouse ELISA (CrystalChem Inc, Chicago, IL) as per the manufacturer's instructions. In brief, 45µl of diluent were added to the antibody coated wells along with 50µl of guinea pig anti-mouse leptin serum. 5µl of plasma (or standard) was added and incubated for twenty hours at 4ºC. After washing with buffer (x5) 100µl of the anti-guinea pig IgG enzyme conjugate solution was added to each well, incubating for 3 hours at 4ºC. After additional washes (x7) 100µl of the enzyme substrate solution was added to each well and incubated for 30 minutes at room temperature. An enzyme stopping solution was then added and absorbance measured at 450nm (subtracting absorbance at 630nm). Concentrations were calculated from a standard curve prepared from a standard solution. Standards were run in duplicate accepting a variance of <10%, and the standard curve was deemed acceptable if R values were > 99%.

2.11 MURINE SURGICAL TECHNIQUES

2.11.1 Mouse jugular venous cannulation

To enable tracer infusions in live unanaesthetised mice, as in Chapter 4, cannulation of a large central vein is necessary. This can then be tunnelled subcutaneously for

externalisation after the mouse has recovered from the procedure. I learned this method from a local investigator with previous experience of the technique and describe the method in detail below.

Mice were anaesthetised using a combination of intraperitoneally administered medetomidine (Domitor, Orion Pharma, Espoo, Finland) and ketamine (Vetalar, Boehringer Vetmedica Inc, MO, USA) and jugular venous cannulae sited as described below with the aid of an operating microscope.

After assessing that the mouse was adequately anaesthetised, a collar of hair was shaved around the neck and the area sterilised with alcohol. Lacrilube ointment was then applied to both cornea to prevent abrasions during surgery. The mouse was placed prone on a heat pad and covered with clingfilm to help maintain body temperature during surgery.

An approximately 1cm long incision was cut from the angle of the jaw to the clavicle on the right hand side and superficial fat dissected to reveal the jugular vein. Fine forceps were used to pass sutures (5-0 Mersilk, Ethicon, Johnson and Johnson, Langhorne, PA, USA) under the rostral and caudal ends of the jugular vein. An additional looped suture was passed through the mid-section which was then cut at the looped end to provide two additional sutures to secure the cannula. The rostral stitch was first tied to occlude venous inflow and both rostral and caudal stitches anchored using haemostat clamps (Fine Science Tools, Heidelberg, Germany) creating tension on the vessel.

A length of Microrethane implantation tubing (0.025" O.D. x 0.012" I.D, Sandown Scientific, Middlesex, UK) was “locked” with 1:10,000 heparin solution. The vein was punctured using an angled 25 gauge needle and the cannula inserted into the vein whilst removing the needle tip. The cannula was advanced approximately 0.5cm and aspirated to confirm correct position and securely fixed in place using the sutures and tissue adhesive (Vetbond, 3M, MN, USA).

The cannula was cut to approximately 8cm in length, tied in a knot at its distal end, and a suture tied beneath the knot leaving 3-4cm of suture which would later be used to locate the cannula. A small incision was cut on the back of the neck and the cannula externalised through the incision prior to being burrowed subcutaneously along the dorsum of the mouse. The stitch ends were left external to the incision. Surgical clips were used to close the dorsal and neck wounds.

Antipamezole (Antisedan, Pfizer AH, Kent, UK), anaesthetic reversal agent, and analgesia, buprenorphine (Vetergesic, Alstoe Animal Health, Leics, UK) were administered and mice were placed on a heat mat to recover. Mice were allowed 3-4 days to recover prior to being studied.

3 Chapter 3-Susceptibility to steatohepatitis and hepatic fibrosis in choline ± methionine deficient models of fatty liver in mice

3.1 INTRODUCTION

Associated with obesity and type 2 diabetes, there is increasing public health concern over the long term consequences of non-alcoholic fatty liver disease (NAFLD). Present in up to one third of the US population (Szczepaniak et al. 2005), NAFLD encompasses a spectrum of disease, and whereas simple steatosis may be relatively benign, hepatic inflammation (non-alcoholic steatohepatitis, NASH) may ensue, potentially progressing to hepatic fibrosis, and its end stage, cirrhosis, with an associated increased risk of hepatocellular carcinoma.

The factors influencing both the development and progression of NAFLD are incompletely defined, but abnormalities of fatty acid metabolism may be critical. Fatty acids are inherently toxic to hepatocytes and hepatic steatosis may represent a protective mechanism, acting as an acute store, “mopping up” the overspill of toxic fatty acids in obesity (Yamaguchi et al. 2007). However, when hepatic triglyceride storage, and/or VLDL export and mitochondrial beta oxidation are overwhelmed, then excess fatty acids are directed towards alternative, less efficient, cytochrome P450 (CYP2E1 and CYP4A) dependent pathways which generate reactive oxygen species (ROS), promoting inflammation, which may lead to fibrosis (Robertson et al. 2001).

Diets deficient in methyl donors such as methionine and/or choline, important in phosphatidyl choline synthesis, reliably induce fatty liver in rodents (Nakae 1999). A diet deficient in both methionine and choline is commonly employed as a model of steatohepatitis with hepatic insulin resistance (Leclercq et al. 2007;Schattenberg et al. 2005), although associated weight loss improves peripheral insulin sensitivity (Rinella and Green 2004). In contrast, a simple choline deficient diet, with adequate methionine, leads to generalised steatosis without obvious inflammation, and

improves insulin sensitivity in mice fed a high fat diet, which may relate to differences in intra-hepatic fatty acid metabolism (Raubenheimer et al. 2006). In addition to hepatic inflammation, methionine and choline deficiency can also lead to mild hepatic fibrosis and, although not previously studied in mice, prolonged administration of a choline deficient diet in rats results in hepatic cirrhosis (Mu et al. 2010;Sakaida et al. 1998).

A carbon tetrachloride (CCl_4) toxin induced model is arguably the most widely studied and best characterised model of liver fibrosis. CCl_4 is rapidly metabolised to a trichloromethyl radical (CCl_3*) by cytochrome P450 (CYP) 2E1, CYP2B and possibly CYP3A, leading to hepatic necrosis (Goodman 1980;Yamazaki et al. 2005). Repeated administration by intraperitoneal injection reliably leads to induction of hepatic fibrosis and (following more prolonged administration) cirrhosis in rodents (Constandinou et al. 2005).

We aimed to establish whether CDD and MCDD dietary models of NAFLD in mice have a contrasting predisposition to hepatic fibrosis as well as their reported contrasting inflammation, and to dissect the cellular and molecular basis underlying any observed differences.

3.2 RESEARCH DESIGN & METHODS

3.2.1 Materials

All chemicals were purchased from Sigma (Poole, UK) unless otherwise stated and solvents were glass-distilled HPLC grade (Rathburn, Walkerburn, UK).

3.2.2 Induction of liver fibrosis

All experiments were carried out under a UK Home Office animal license using 12 week old male C57BL/6J mice (Harlan, Bicester, UK) maintained under controlled

conditions of light and temperature. Following a 2 week period of acclimatisation, mice (n=7/group) were offered either control (choline and methionine supplemented) chow (CS), choline deficient diet (CDD) or methionine-choline deficient diet (MCDD) (Dyets Inc., Bethlehem, PA) for 4 weeks and treated with twice weekly intraperitoneal injections of 0.3µl/g CCl_4 (1:3 v/v in olive oil) or olive oil alone as controls. Injections were performed in a ventilated fume hood, using a gas-tight Teflon lined 100µl glass syringe (Hamilton, Bonaduz, Switzerland) and disposable 25 gauge needles. Mice were sacrificed by decapitation 3 days following the last CCl_4 injection, being culled in the *ad libitum* fed state. Trunk blood was collected and sections of liver fixed in 10%v/v formalin. The remainder of the liver was snap frozen and stored at -80ºC prior to analysis.

3.2.3 Histology and immunohistochemistry

Liver sections were processed and stained as described in Section 2.5.

3.2.4 Histological characterisation

3.2.4.1 Quantification of liver fibrosis

Picosirius red, which stains polymeric and monomeric collagen matrix, was used to quantify hepatic fibrosis. 30 randomly selected high power field (200x) photographs were captured per liver section using Axiovision software, and AxioCam MRc digital camera, attached to an Axiovert 200 microscope (Zeiss, Germany) and analysed in a blinded manner using commercial software (Adobe photoshop CS3 version 10.0, Adobe systems Inc., San Jose, CA, USA) to provide a continuous variable representing hepatic fibrosis. Pixels staining red were identified, divided by the total number of pixels per photograph and multiplied by 100, to give a percentage positive staining for collagen. To avoid confounding, sections containing large blood vessels were avoided.

3.2.4.2 Cell counts

Cell counts of neutrophils and activated hepatic stellate cells (αSMA stain) were undertaken in a blinded manner, averaging the number of cells in 20 representative high power fields per liver section.

3.2.4.3 Macrophage activation

Given the relatively uniform staining of hepatic macrophages, image analysis software was used to determine F4/80 staining in a manner analogous to that described for picosirius red.

3.2.5 Messenger RNA (mRNA) extraction and quantification

mRNA expression of pro-inflammatory and pro-fibrogenic genes was assessed using real time PCR as described in detail in section 2.6. Primers and probes were designed from the Roche UPL library and are listed in Table 3.1. The relative amount of mRNA was derived from standard curves prepared from serial dilutions of cDNA and transcript levels were normalised to mean values of an internal control house keeping gene (GAPDH, Assay ID Mm99999915_g1, Applied Biosystems, Warrington, UK).

Table 3.1 Primers and probe details.

mRNA of Gene	Genbank RefSeq	Sequence	Direction	UPL probe no.
Il1b	NM_008361.3	ttgacggaccccaaaagat	Forward	38
		agctggatgctctcatcagg	Reverse	
Tnf	NM_013693.2	tgcctatgtctcagcctcttc	Forward	49
		gaggccatttgggaacttct	Reverse	
Ccl2 (Mcp-1)	NM_011333.3	gcctgctgttcacagttgc	Forward	102
		caggtgagtggggcgtta	Reverse	
Tgfb1	NM_011577.1	tggagcaacatgtggaactc	Forward	72
		cagcagccggttaccaag	Reverse	
Col1a2	NM_007743.2	gcaggttcacctactctgtcct	Forward	46
		cttgccccattcatttgtct	Reverse	
Acta2 (Sma2)	NM_007392.2	gacaccacccacccagagt	Forward	20
		acatagctggagcagcgtct	Reverse	
Mmp2	NM_008610.2	taacctggatgccgtcgt	Forward	77
		ttcaggtaataagcacccttgaa	Reverse	
Mmp9	NM_013599.2	acgacatagacggcatcca	Forward	19
		gctgtggttcagttgtggtg	Reverse	
Mmp12	NM_008605.3	ttgtggataaacactactggaggt	Forward	51
		aaatcagcttggggtaagca	Reverse	
Mmp13	NM_008607.1	cagtctccgaggagaaactatgat	Forward	62
		ggactttgtcaaaaagagctcag	Reverse	
Timp1	NM_001044384.1	gcaaagagctttctcaaagacc	Forward	76
		agggatagataaacagggaaacact	Reverse	
Timp2	NM_011594.3	cgttttgcaatgcagacgta	Forward	21
		ggaatccacctccttctcg	Reverse	

3.2.6 Liver triglycerides

Liver TGs were measured using a commercial triglyceride kit (ThermoTrace, Australia) as described in Section 2.10.3.

3.2.7 Leptin assay

Plasma leptin levels were measured using an ultrasensitive mouse ELISA (CrystalChem Inc, Chicago, IL) as described in Section 2.10.7.

3.2.8 Statistical analysis

Results are presented as mean ± standard error (SEM). Between groups differences were compared by two way ANOVA, with Bonferroni's post hoc multiple comparison test when appropriate, using GraphPad Prism software (La Jolla, CA, USA) and SPSS (IBM, NY, USA).

3.3 RESULTS

3.3.1 Influence of choline ± methionine deficiency on body weights and composition in olive oil treated mice

Starting body weights did not differ between CS, CDD and MCDD groups injected with olive oil twice weekly (Figure 3.1). As expected, MCDD mice lost weight (~30% by 4 weeks) compared to both CS and CDD mice, whilst the final body weights of CDD and CS mice did not differ. Despite weight loss, MCDD mice were otherwise well with no external signs of distress.

Weight loss in MCDD mice was associated with loss of both subcutaneous and perigonadal adipose tissue compared with both CS and CDD mice, whereas there was no difference in adipose tissue weights between CDD and CS mice (Table 3.2). Weight loss in MCDD mice was associated with a lower plasma leptin concentration versus both CS and CDD mice, whilst leptin did not differ between CS and CDD mice (Figure 3.2). In contrast, there was no difference in muscle mass, as a percentage of total body weight, between MCDD and CS or CDD mice, with no additional difference between CDD and CS mice.

Liver TG concentrations were similarly increased in both MCDD and CDD compared to CS fed mice (Table 3.2). Liver weights were lower in MCDD mice versus CS and CDD mice, but were in proportion to total body weights (Table 3.2).

3.3.2 Influence of choline ± methionine deficiency on hepatic inflammation in olive oil treated mice

Immunohistochemistry confirmed an acute inflammatory infiltrate, with increased neutrophil staining in MCDD mice alone, compared to both CS and CDD mice, whilst there was no statistically significant increase in neutrophil staining in CDD mice (Figure 3.3). There was no detectable increase in macrophage staining in either dietary group. This was despite an increased abundance of mRNA trancripts of *Ccl2* (MCP1) in both MCDD and CDD mice (Figure 3.4A) compared to CS controls. mRNA levels of the genes encoding the pro-inflammatory cytokines TNFα and IL-1b were also increased in the MCDD group, versus both CS and CDD mice, and CS mice respectively, whilst there was no significant increase in CDD mice on post hoc analysis.

3.3.3 Influence of choline ± methionine deficiency on hepatic fibrosis in olive oil treated control mice

Quantification of picosirius red staining demonstrated no significant difference in hepatic fibrosis between dietary groups (Figure 3.5), with only a trend (P=0.067) for an influence of diet on picosirius red staining. Diet did influence hepatic stellate cell activation (αSMA staining), with increased αSMA staining in MCDD mice, compared with both CS and CDD mice, with no difference between CDD and CS mice (Figure 3.5). Similarly, the abundance of hepatic transcripts of *Acta2* and *Tgfb1* was increased in MCDD versus CS and CDD mice (Figure 3.4A), whilst there was no significant increase in CDD mice alone. There was no influence of diet on *Col1a* mRNA levels.

MCDD upregulated *Mmp* gene expression, increasing mRNA transcripts of *Mmps* 12, and 13 compared with both CS and CDD mice, whilst *Mmp* 9 mRNA levels were increased versus CS mice alone (Figure 3.4B). In contrast, *Mmp* gene expression was unchanged in CDD mice. *Timp1* and *Timp2* mRNA levels were also increased in MCDD mice versus CS, although not CDD mice, whilst there was no significant increase in expression in CDD mice.

3.3.4 Body weights and composition in CCl_4 treated choline ± methionine deficient mice

Starting body weights were similar in CCl_4 treated CS, CDD and MCDD mice with no differences between their olive oil treated counterparts (Figure 3.1). As with olive oil treated controls, MCDD mice lost weight compared to both CS and CDD groups, but there was no additional effect of CCl_4. In contrast, although the final body weights of CCl_4 treated CS and CDD mice did not differ from each other, CCl_4 treated CS and CDD mice did not gain weight as well as their olive oil treated controls (Figure 3.1).

CCl_4 influenced perigonadal but not subcutaneous fat deposition, with a reduction perigonadal fat in CCl_4 treated CS mice. There was no significant difference in adipose depot weights in CCl_4 treated CDD mice, despite less significant weight gain. CCl_4 did not influence body composition in MCDD mice (Table 3.2). Similarly, CCl_4 had no influence on muscle mass (quadriceps weights as a percentage of body mass) in either dietary group (Table 3.2). Plasma leptin levels were reduced in CCl_4 treated MCDD versus both CS and CDD mice, although there was no additional effect of CCl_4 in either group (Figure 3.2).

Liver TG concentrations remained elevated in both CCl_4 treated MCDD and CDD mice versus CS controls, but there was no additional effect of CCl_4 (Table 3.2). There was a trend ($F[1,36]=3.75$, $P=0.061$) for a reduction in liver weight in response to CCl_4, but there was no change in liver weights expressed as a percentage of final body weight.

3.3.5 Effect of CCl_4 treatment on hepatic inflammation in choline ± methionine deficiency

CCl_4 did not significantly influence hepatic neutrophil staining in any dietary group, although neutrophil counts remained significantly higher in MCDD than both CS and CDD mice (Figure 3.3). There was no detectable influence of CCl_4 treatment on macrophage staining (Figure 3.3). There was no additional effect of CCl_4 treatment on mRNA levels of *Tnf*, *Il1b*, and *Ccl2* (Figure 3.4A).

3.3.6 Effect of choline ± methionine deficiency on sensitivity to CCl_4 induced hepatic fibrosis

Picosirius red staining confirmed that twice weekly injections of CCl_4 induced hepatic fibrosis in all three dietary groups (Figure 3.5). The increase in picosirius red staining in response to CCl_4 was less pronounced in MCDD mice, although there was only a trend for an *interaction* between diet and CCl_4 treatment by two way ANOVA ($F[2,36]=2.81$, $P=0.74$).

CCl_4 induced hepatic stellate cell activation, with post hoc analysis demonstrating a significant increase in αSMA staining in response to CCl_4 in MCDD mice alone (Figure 3.5). However, two way ANOVA did not demonstrate any *interaction* between diet and CCl_4 treatment.

Unlike the immunohistochemistry results, there was no additional effect of CCl_4 on mRNA levels of *Acta2* (encoding αSMA) (Figure 3.4). In addition, CCl_4 did not influence *Col1A* or *Tgfb1* mRNA levels.

CCl_4 treatment did not influence expression of *Mmp* 9, 12 and 13, or *Timp* 1 (Figure 3.4B). There was an effect of CCl_4 on *Timp2* mRNA levels, although post hoc tests did not reveal any significant differences. CCl_4 also influenced mRNA transcripts of *Mmp2*, although post hoc testing showed a significant difference in CDD mice alone.

Figure 3.1 Effect of choline ± methionine deficiency and carbon tetrachloride treatment on body weight

A) Body weights in olive oil (OO) treated controls and B) CCl_4 treated CS, CDD and MCDD fed mice. Data are mean ± SEM (N=7/group). Final body weights were analysed by two way ANOVA with Bonferonni`s post hoc test where appropriate. There was a significant influence of diet on final body weights F(2,36)=154.15, P<0.0001, with P<0.0001 for MCDD vs CDD and CS mice. Carbon tetrachloride (CCl_4) also had a significant influence on final body weights F(1,36)=16.47, P<0.001; P<0.05 for CCl_4 treated CS mice, and P<0.01 for CCl_4 treated CDD mice. There was a significant interaction between diet and CCl_4 F(2,36)=3.99, P=0.027 and simple main effects analysis found an effect of CCl_4 on final body weights in CS (F=9.42, P<0.01) and CDD (F=14.97, P<0.001) mice.

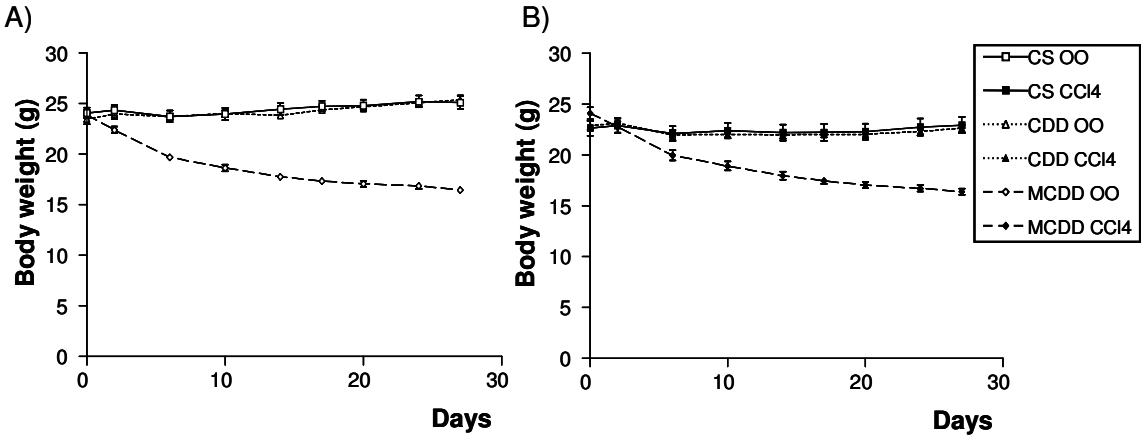


Figure 3.2 Leptin levels in choline ± methionine deficiency

Results are mean ± SEM. Differences between groups were analysed by two way ANOVA. N=6-7/group. The was a significant influence of diet on plasma leptin levels F(2,35)=15.98, p<0.0001. P<0.0001 for MCDD vs CS, P<0.001 for MCDD vs CDD using Bonferonni's post hoc test.

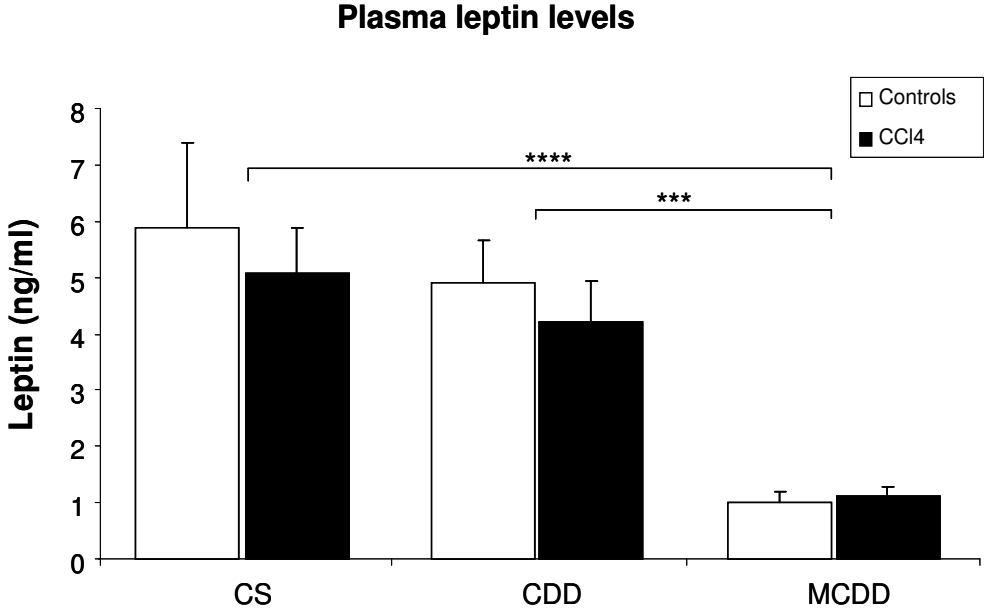


Table 3.2 Influence of choline ± methionine deficiency and carbon tetrachloride (CCl_4) treatment on body composition

Data are mean ± SEM. N=7/group. Differences between groups were analysed by two way ANOVA with Bonferroni's post hoc tests where appropriate. *****F(2,36)=24.51, P<0.0001, for an effect of diet on liver weights; P<0.0001 for MCDD vs CS and CDD mice.* ††††*F(2,36)=20.38, P<0.0001 for an effect of diet on liver triglyceride (TG) concentrations; P<0.0001 for CDD vs CS and MCDD vs CS.* §§§§*F(2,36)=54.81, P<0.0001 for an effect of diet on gonadal fat; P<0.0001 for MCDD vs CS and CDD mice, and* §§§*F(1,36)=8.61, P=0.006 for an effect of CCl_4 on gonadal fat*; *P<0.05 for CCl_4 treated CS mice.* ‡‡‡‡F*(2,36)=21.44; P<0.0001 for an effect of diet on subcutaneous adipose tissue; P<0.0001 for MCDD vs CS and CDD mice.*

	Controls			CCl_4		
	CS	CDD	MCDD	CS	CDD	MCDD
Liver						
Weight (g) ****	1.29±0.05	1.28±0.09	0.87±0.03	1.21±0.08	1.14±0.1	0.78±0.03
Weight (% body weight)	5.1±0.1	5.1±0.3	5.3±0.2	5.2±0.2	5.0±0.4	4.7±0.1
Liver TGs (µmol/g) ††††	19.6±4.1	34.4±2.6	39.9±2.2	17.4±2.8	40.2±5.4	40.3±4.0
Fat depots (as % body weight)						
Gonadal §§§§/§§§	1.7±0.1	1.7±0.2	0.5±0.1	1.3±0.1	1.4±0.1	0.4±0.1
Subcutaneous ‡‡‡‡	2.4±0.3	2.4±0.2	1.3±0.1	2.4±0.1	2.2±0.1	1.4±0.1
Muscle weight (as % body weight)						
Quadriceps	0.62±0.03	0.61±0.02	0.61±0.01	0.62±0.04	0.60±0.04	0.69±0.01

Figure 3.3 Hepatic neutrophil and macrophage staining in carbon tetrachloride treated choline ± methionine deficient mice

A) Hepatic neutrophil staining (anti GR1 antibody) and B) cells counts from male C57Bl6 mice fed a CS, CDD or MCDD and treated with carbon tetrachloride (CCl_4) or olive oil (OO). C) F4/80 staining of hepatic macrophages, and D) quantification by pixel analysis. Images x200 magnification. Results are mean ± SEM. N=7/group. Differences between groups were analysed by two way ANOVA with Bonferroni's post hoc analysis where appropriate. There was a significant effect of diet on neutrophil counts F(2,36)=19.70, P<0.0001; P<0.0001 for MCDD vs CS, P<0.001 for MCDD vs CDD.

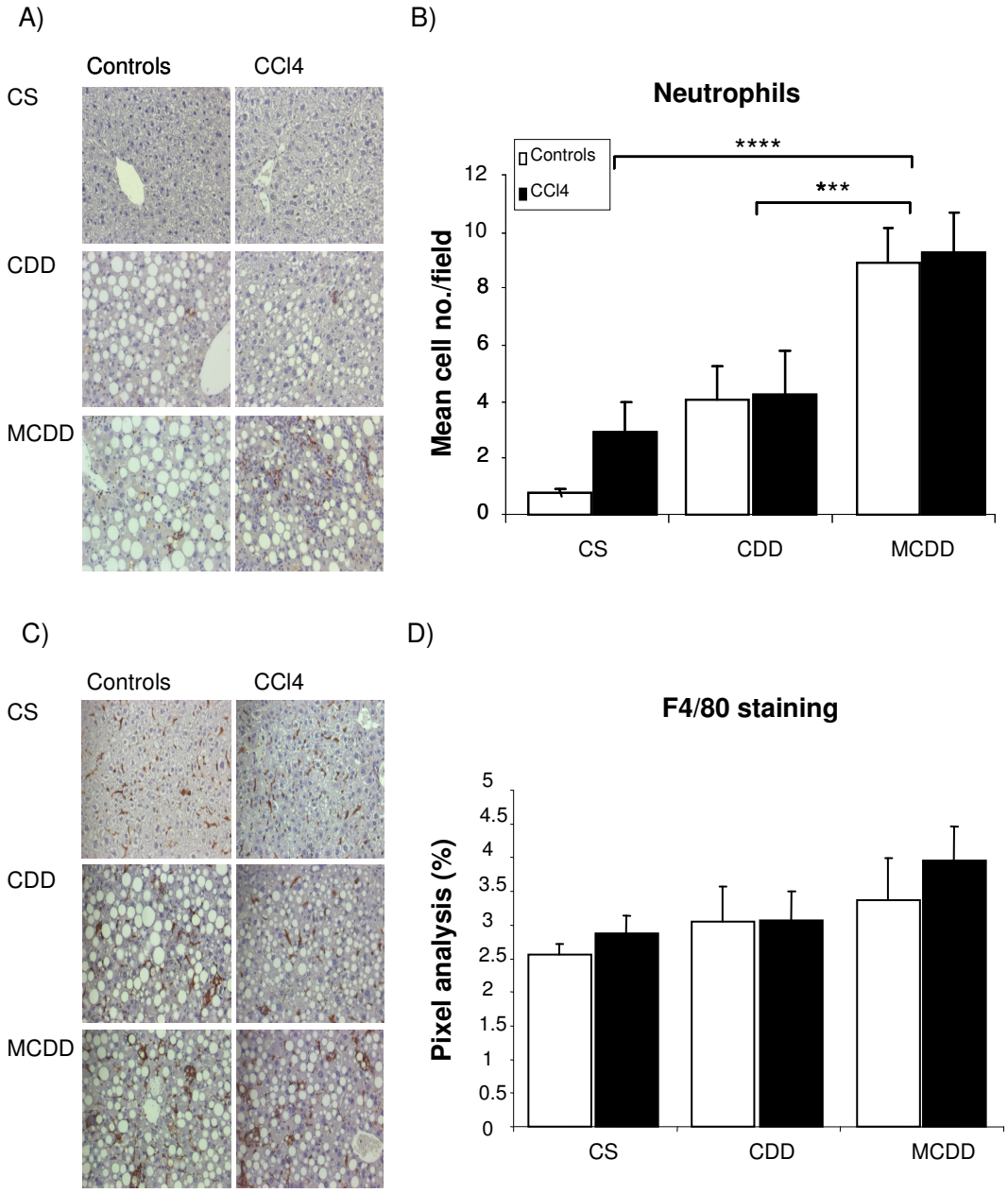
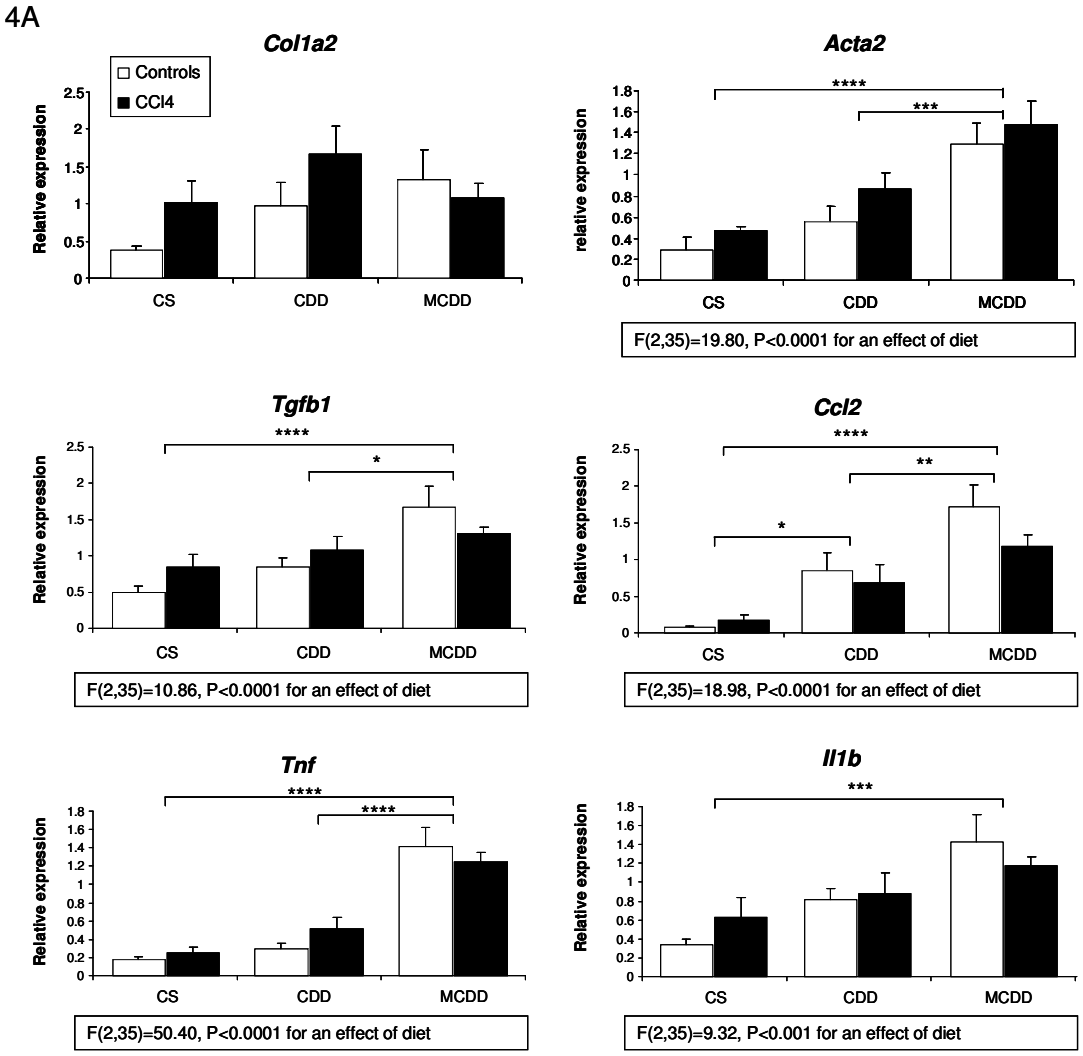


Figure 3.4 Influence of choline ± methionine deficiency and carbon tetrachloride on hepatic gene expression

Abundance of gene transcripts are expressed relative to that of internal control genes. Transcripts of proinflammatory and profibrotic genes are presented in panel A and matrix metalloproteinase (Mmp) and tissue inhibitors of matrix metalloproteinases (Timps) in panel B. Data are expressed as a ratio to the abundance of transcripts of control gene, as means ± SEM. N=5-6/group. Differences between groups were analysed by two way ANOVA with Bonferroni's post hoc comparison test where appropriate.



4B

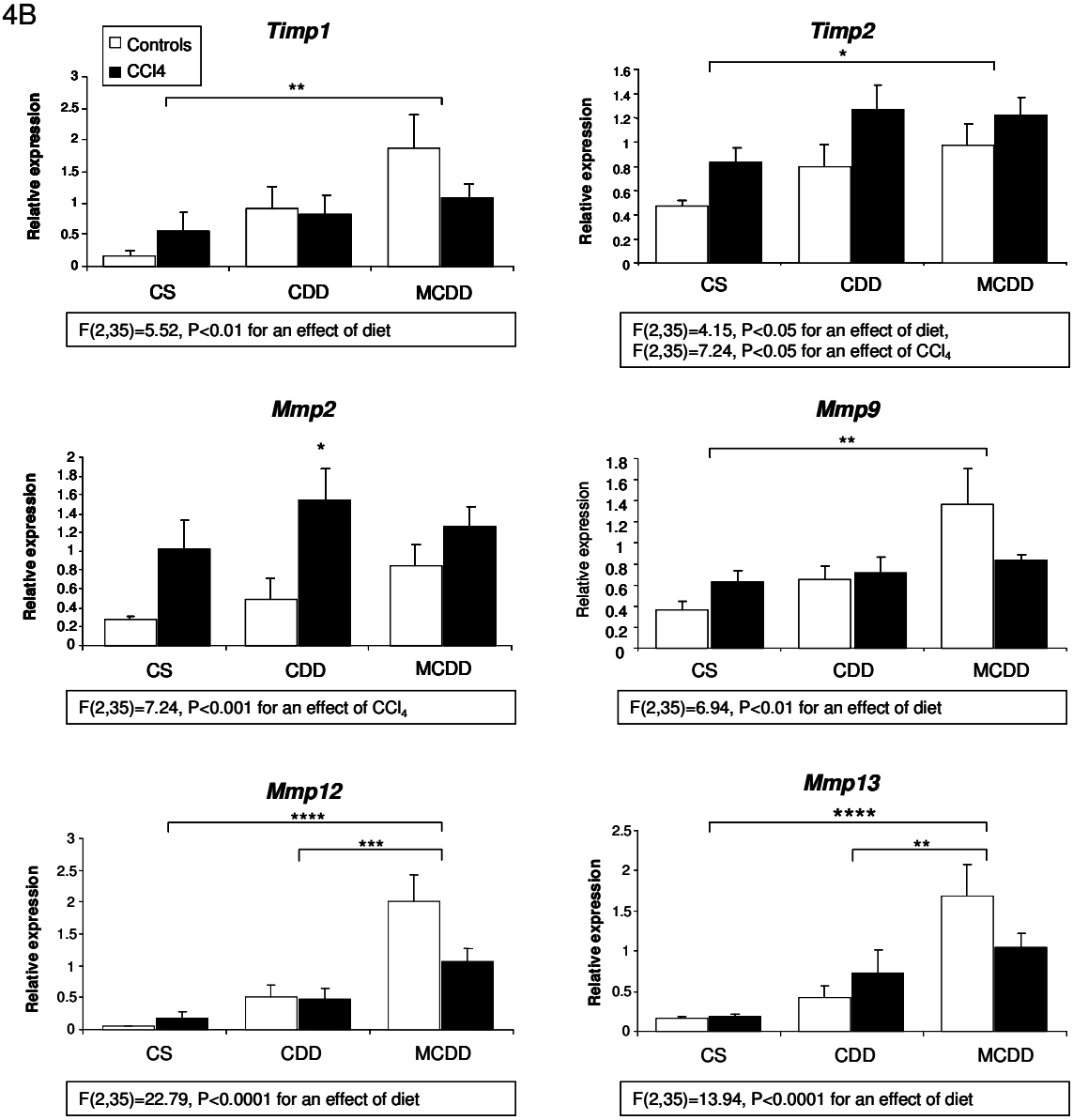
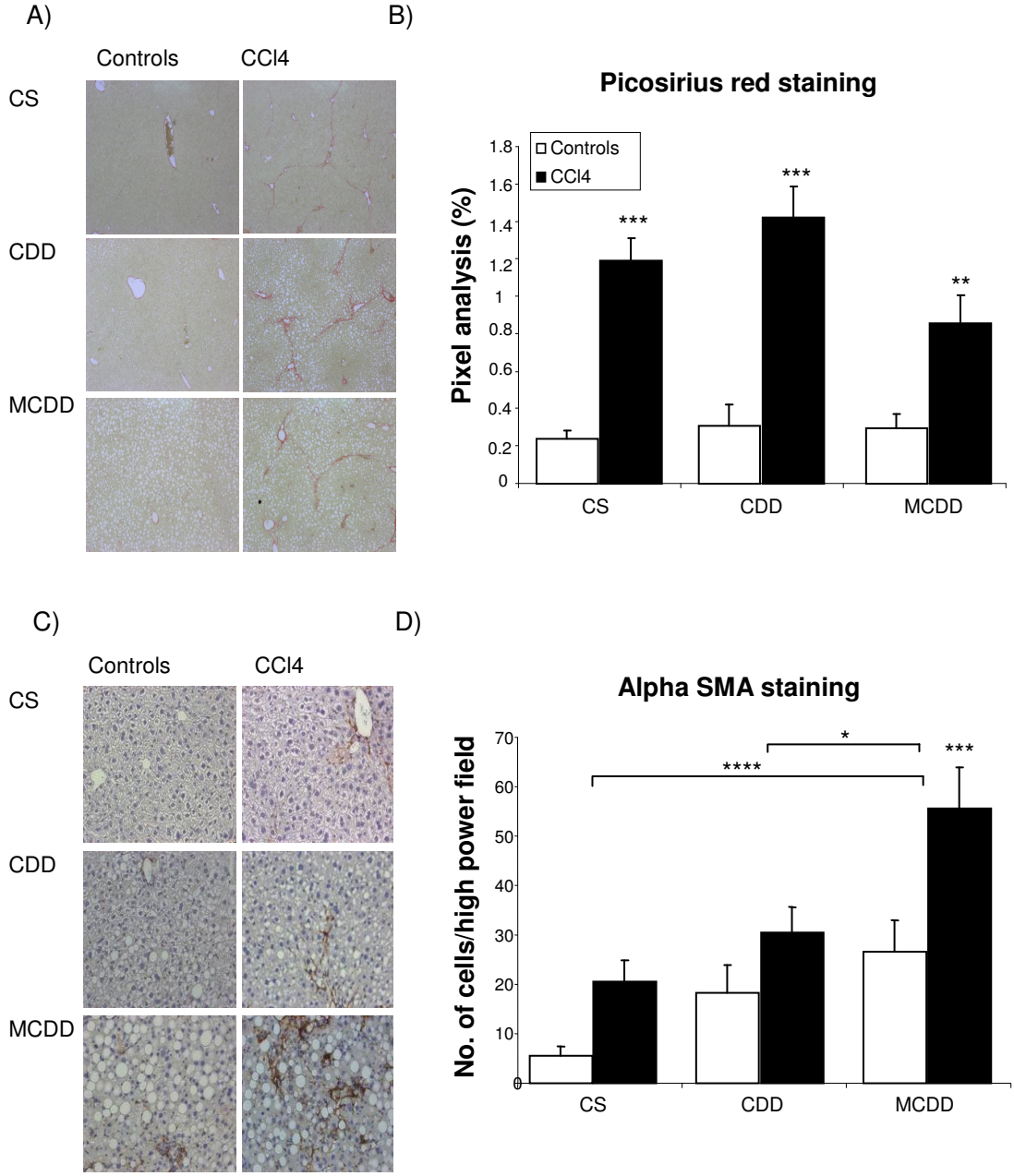

Timp1
Controls
CCl4
Relative expression
**
CS
CDD
MCDD
F(2,35)=5.52, P<0.01 for an effect of diet
Timp2
*
F(2,35)=4.15, P<0.05 for an effect of diet,
F(2,35)=7.24, P<0.05 for an effect of CCl4
Mmp2
*
F(2,35)=7.24, P<0.001 for an effect of CCl4
Mmp9
**
F(2,35)=6.94, P<0.01 for an effect of diet
Mmp12
****
***
F(2,35)=22.79, P<0.0001 for an effect of diet
Mmp13
****
**
F(2,35)=13.94, P<0.0001 for an effect of diet

Figure 3.5 Hepatic fibrosis and hepatic stellate cell activation in carbon tetrachloride treated choline ± methionine deficient mice

A) Picosirius red staining (PSR) in CS, CDD, and MCDD mice treated with olive oil (OO) or carbon tetrachloride (CCl_4). Magnification x50. B) PSR image analysis C) αSMA staining and D) hepatic stellate cells per high power field (D). Magnification x200. Results are mean ± SEM. N=7/group. Differences between groups were analysed by two way ANOVA with Bonferroni's post hoc analysis where appropriate. F(1,36)=79.66, P<0.0001 for an effect of CCl_4 on PSR staining. F(2,36)=12.68, P<0.0001 for an effect of diet on αSMA staining. F(1,36)=16.86, P<0.0001 for an effect of CCl_4 on αSMA staining



3.4 DISCUSSION

Liver histology and immunohistochemistry demonstrated an acute inflammatory infiltrate in mice fed the methionine and choline deficient diet (MCDD), confirming that MCDD, and not an isolated choline deficient diet (CDD), leads to significant steatohepatitis after four weeks. However, the data demonstrates that steatohepatitis secondary to MCDD does not increase susceptibility to carbon tetrachloride (CCl_4) induced liver fibrosis in mice. Indeed, CCl_4 induced hepatic fibrosis was, if anything, lower in MCDD fed mice than in those with simple steatosis due to choline deficiency alone. These findings emphasise the complexity of the relationship between hepatic inflammation and fibrosis, suggesting that factors additional to those promoting steatohepatitis may determine progression to hepatic fibrosis.

In addition to steatohepatitis, the MCDD, but not the CDD, led to activation of hepatic stellate cells (HSCs), central players in the development of hepatic fibrosis. Furthermore, the MCDD increased both pro-inflammatory and pro-fibrotic cytokine gene expression, including *Tnf*, *Il1b*, and *Tgfb1*. Other key baseline differences between dietary models included the expression of the genes encoding the matrix metalloproteinases (MMPs), and their tissue inhibitors (TIMPs). The MCDD increased expression of *Mmp* 9, 12 and 13, and *Timp1*, although despite the latter there was no detectable increase in fibrosis by analysis of picosirius red staining.

Picosirius red staining confirmed hepatic fibrosis in each dietary group treated with CCl_4, compared with olive oil treated controls. However, despite the greater CCl_4-associated activation of hepatic stellate cells in MCDD than CDD mice, and regardless of the MCDD induced pro-inflammatory and pro-fibrotic phenotype, there was no increase in fibrosis in MCDD than CDD treated mice. Furthermore, despite generalised upregulation of MMPs in MCDD mice, they did not upregulate *Mmp2* expression as was seen in CDD mice following CCl_4 injury.

Current dogma suggests that chronic liver injury and therefore inflammation leads to hepatic fibrosis, with cytokines such as those upregulated by the MCDD, leading to

HSC activation and increased production of TIMPs. Hepatic stellate cells are central to the development of hepatic fibrosis, developing a myofibroblast-like phenotype in response to inflammation, and secreting the fibrillar collagens that contribute to the fibrotic extracellular matrix (ECM) (Atzori et al. 2009;Rockey et al. 1992). The HSCs can also produce MMPs/TIMPs, cytokines (eg TGFβ), and oxidative stress (Atzori et al. 2009).

The increase in hepatic stellate cell activation was marked in MCDD mice. However, this was not translated into increased fibrosis, suggesting that factors distal to hepatic stellate cell activation in the complex fibrotic cascade are essential for translation into hepatic fibrosis. The increased extracellular matrix deposition in liver fibrosis can be considered an imbalance between matrix deposition and degradation, with MMPs and their inhibitors playing an essential role in this process. Although pro-fibrogenic TIMP1 was upregulated in MCDD mice, the marked MMP response in may have hastened the resolution of any CCl_4 induced scar in this group. Indeed, the lack of upregulation of *Col1a2* suggests that collagen synthesis was occurring at similar rates in all groups, supporting the hypothesis that altered rates of degradation may be a key difference between these models. However, the dynamic nature of MMP and TIMP expression also means that we may have missed early differences three days after CCl_4 injection, and may explain for example the differences between αSMA staining and gene expression (Hemmann et al. 2007).

Clearly a key difference between the groups was the weight loss in the MCDD group. This was associated with low leptin levels in the MCDD but not the CDD group, and the weight loss in the MCDD group may therefore be acting to protect the liver from fibrosis. In keeping with this, obese, diabetic, hyperleptinaemic *db/db* mice fail to lose weight and develop increased hepatic fibrosis compared to *ob/ob* and *db/m* controls fed a MCDD. Leptin appears to be essential for the fibrogenic response to CCl_4 (Leclercq et al. 2002). Another adipocytokine, adiponectin, may also be important in the progression to fibrosis, having previously been shown to be increased in the MCDD model (Okumura et al. 2006), and adiponectin can inhibit hepatic stellate cell activation (Adachi et al. 2008).

No rodent model of NAFLD fully recapitulates human disease. Nonetheless, MCDD has been extensively studied in rodents as it reliably produces a histological picture similar to human non-alcoholic steatohepatitis (NASH), and prolonged MCDD feeding can induce mild fibrosis (Mu et al. 2010;Sahai et al. 2004a). Previous studies have confirmed upregulation of pro-inflammatory and pro-fibrotic cytokines in MCDD mice (e.g. (Leclercq et al. 2004;Mu et al. 2010;Sahai et al. 2004b)). Similarly, increased TIMP1 and MMP expression has recently been demonstrated in the MCDD model (Mu et al. 2010). However, crucially, previous studies have not included controls with fatty liver. A CDD leads to steatosis alone and has been less well studied than MCDD in fatty liver research, although prolonged administration in rats leads to hepatic fibrosis (Mu et al. 2010;Sakaida et al. 1998). Intriguingly CDD improves insulin sensitivity in high fat fed mice, possibly due to altered intra-hepatic fatty acid handling (Raubenheimer et al. 2006). Whether these differences between CDD and MCDD mice influence susceptibility to hepatic fibrosis has not previously been studied.

Carbon tetrachloride injury is probably the best characterised model of hepatic fibrogenesis. CCl_4 is metabolised to toxic trichloromethyl (CCl_3*) free radicals by cytochrome P450s (especially CYP2E1, 2B1 and CYP3A) (Clawson 1989), which reacts with oxygen to form trichloromethylperoxy radicals (CCl_3OO*) causing lipid peroxidation and possibly reducing VLDL export (Boll et al. 2001;Weber et al. 2003). Indeed, given the upregulation of CCl_4 metabolising CYP2E1 in MCDD the model (Leclercq et al. 2000), and the proposed role of oxidative stress in the pathogenesis of the MCDD model (Oz et al. 2006), we had hypothesised a greater fibrotic reaction in the MCDD group. Reduced phosphatidlycholine synthesis in MCDD mice may partly explain this finding as CCl_3* has been shown to bind phosphatidylcholine in particular (Boll et al. 2001). Interestingly, we did not see conversion of the CDD into MCDD phenotype, which might have been expected if a lack of hepatic methionine/glutathione dependent antioxidants was the only difference between MCDD and CDD fed rodents (Oz et al. 2006), supporting the hypothesis that factors other than oxidative stress explain the contrasting phenotypes

in these two models. In support of this, markers of oxidative stress do not increase until after eight weeks in MCDD fed mice, with mild fibrosis not occurring until this time (Sahai et al. 2004a;Yamaguchi et al. 2007)

It is possible the duration of the experiment may have limited our ability to detect differences in fibrosis between groups. However, we were unable to study mice for longer than in the current experiment due to UK Home Office limitations with respect to weight loss, although four weeks of CCl_4 treatment is usually sufficient to induce fibrosis (Constandinou et al. 2005). Lastly, it is possible that our method of quantifying fibrosis may have underestimated the degree of perivascular fibrosis in MCDD mice, as we avoided large blood vessels to avoid confounding effects of vascular type 1 collagen staining. Additional measures of hepatic fibrosis e.g. semi-quantitative histological scoring or hepatic hydroxyproline content analysis may also have been helpful.

In summary, we examined the susceptibility of two contrasting dietary models of NAFLD and demonstrated that in mice deficient in choline and/or methionine neither steatosis nor steatohepatitis increased susceptibility to CCl_4 induced fibrosis. This was despite greater upregulation of pro-inflammatory and pro-fibrotic cytokines, and more marked hepatic stellate cell activation in MCDD mice. The significant upregulation of matrix turnover apparatus in the MCDD model may have limited the fibrogenic response by enhancing collagen degradation. In addition, the reduction in leptin levels was another key difference potentially limiting fibrosis in MCDD fed mice, supporting the hypothesis that the development of progressive liver disease is dependent on the associated metabolic milieu. Two inter-related metabolic factors that may be critical in modulating progression to liver disease include intrahepatic fatty acid and glucocorticoid metabolism. These factors may have important implications for human disease, potentially explaining the highly variable progression to fibrosis in NAFLD.

4 Chapter 4-Metabolic pathways promoting intrahepatic fatty acid accumulation in methionine and choline deficiency; implications for the pathogenesis of steatohepatitis

4.1 INTRODUCTION

In Chapter 3 I confirmed that mice fed a methionine and choline deficient diet (MCDD), but not a choline deficient diet (CDD), developed a pro-inflammatory and pro-fibrotic phenotype associated with the development of steatohepatitis. The mechanisms underlying susceptibility to progressive liver disease in the MCDD model, and in NAFLD in general, remain incompletely defined. Importantly, it is not known whether specific changes in hepatic triglyceride handling leading to the accumulation of intracellular fatty acids predisposes to hepatocyte damage and the development of non-alcoholic steatohepatitis (NASH). However, the marked weight loss induced by the MCDD would suggest that changes in fatty acid metabolism may be central to the development of NASH in this model.

Broadly speaking, the accumulation of hepatic triglyceride can be considered an imbalance between the supply of free fatty acids (FFAs), from both plasma and intra-hepatic *de novo* lipogenesis (DNL), and their removal either by fatty acid oxidation or export in very low density lipoprotein (VLDL). Studies in humans suggest that both increased release of FFAs from extra hepatic tissues and increased DNL contribute to the accumulation of liver triglyceride in NAFLD (Donnelly et al. 2005;Fabbrini et al. 2008;Fabbrini et al. 2009). Such abnormalities of fatty acid metabolism may play a role not only in the insulin resistance associated with NAFLD, but also in promoting hepatic inflammation. In addition to influencing rates of hepatic gluconeogenesis and VLDL export (Chen et al. 1999;Lewis et al. 1995), FFAs are inherently toxic to hepatocytes (Malhi et al. 2006), e.g. activating the proinflammatory cytokine nuclear transcription factor kappa β (Feldstein et al. 2004;Itani et al. 2002), and upregulating mediators of apoptosis (Feldstein et al. 2003). A toxic role for FFAs in the liver is supported by the observation that

inhibiting diacylglycerol acyltransferase 2, the final enzyme in the pathway esterifying fatty acids to triglycerides, promotes steatohepatitis and liver fibrosis in mice, despite reducing intrahepatic triglycerides (Yamaguchi et al. 2007).

In humans practical limitations mean that only the broadest association can be made between hepatic triglyceride/fatty acid handling and NASH. In contrast the mechanisms underlying susceptibility to steatohepatitis and fibrosis mediated by fatty acids can be defined by studies in animal models with contrasting susceptibility to NASH. Feeding rodents diets deficient in choline and/or methionine reliably leads to the accumulation of intrahepatic triglyceride (Ghoshal and Farber 1993;Nakae 1999), but there are contrasting consequences for liver inflammation. A MCDD causes steatohepatitis and hepatic insulin resistance (Leclercq et al. 2007;Rinella et al. 2008;Rizki et al. 2006), albeit that peripheral insulin sensitivity is improved due to significant weight loss (Rinella and Green 2004). In contrast, a simple CDD leads to steatosis without significant inflammation, and is associated with improved hepatic and peripheral insulin sensitivity without weight loss following a high fat diet (Raubenheimer et al. 2006). These diets provide readily tractable models to define the differences in hepatic fatty acid metabolism that underlies susceptibility to NASH as opposed to simple steatosis.

Impaired VLDL export was previously thought to be the dominant mechanism for the accumulation of liver fat in both CDD and MCDD models (Lombardi et al. 1968). More recent *ex vivo* studies have shown that VLDL export may not be decreased in CDD mice (Kulinski et al. 2004), while alterations in gene expression support the hypothesis that a primary increase in fatty acid esterification protects CDD mice against the toxic effects of intrahepatic fatty acids (Raubenheimer et al. 2006). Moreover, weight loss in MCDD but not CDD mice suggests that increased flux of potentially toxic FFAs from the periphery to the liver may only occur with MCDD. However, these inferences concerning fatty acid metabolism in MCDD and CDD mice are drawn indirectly from *ex vivo* measurements. In this Chapter I have characterized fatty acid metabolism in detail *in vivo* using stable isotope tracers in mice fed CDD and MCDD, and identified pathways which could explain contrasting

propensity to hepatocyte damage and steatohepatitis in these models; observations with implications for understanding the pathogenesis of human NASH.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Stable-isotope labeled tracers were from Cambridge Isotopes (Andover, MA, USA) and all other chemicals from Sigma (Poole, UK) unless otherwise stated. Solvents were glass-distilled HPLC grade (Rathburn, Walkerburn, UK). Human albumin solution (20%w/v) was from the Scottish National Blood Transfusion Service (Lothian, UK).

4.2.2 Animals and procedures

All experiments were carried out under a UK Home Office animal license. 12 week old male C57BL/6J mice (Harlan, Bicester, UK) were maintained under controlled conditions of light and temperature. Following a 2 week period of acclimatization, mice were offered either control (choline and methionine supplemented) chow (CS), choline deficient diet (CDD) or methionine-choline deficient diet (MCDD) (Dyets Inc., Bethlehem, PA) for 2 weeks as per Section 2.2.

4.2.3 Fatty acid flux studies

To quantify FFA turnover by stable isotope dilution, 1,2,3,4-[$^{13}C]_4$-palmitate was infused intravenously (n= 12-15 mice/group) and tracer enrichment of the palmitate pool in liver, adipose tissue and plasma was measured by gas chromatography mass spectrometry (GCMS) (Baar et al. 2005) as described in Section 2.9.3.

Jugular venous cannulae were inserted under general anaesthesia (intraperitoneal medetomidine and ketamine) on day 10 of dietary manipulations, and mice allowed

four days to recover prior to tracer infusions as described in detail in Section 2.11.1. $[^{13}C]_4$ palmitate was prepared by dissolving its potassium salt in saline and complexing with 20% w/v albumin solution at 40°C. In initial studies a stock solution was diluted so that infusion at 5µl/min provided 0.271µmoles/kg/min tracer (Baar et al. 2005;Jung et al. 1999). Following preliminary analysis, the concentration was later increased so that the infusion delivered 0.542µmoles/kg/min to increase tissue enrichment. Calculated kinetic results were similar in both groups so data were combined for statistical analysis. Following a four hour fast, the tracer infusion was commenced using a Harvard apparatus syringe pump (World Precision Instruments, Stevenage, UK) for 180minutes (Jung et al. 1999). Animals were sacrificed by decapitation within 60seconds of being disturbed and trunk blood collected on wet ice. Plasma was stored at -80^{o}C. Liver and adipose depots were immediately harvested, weighed and snap frozen on dry ice. Palmitate and its tracer were quantified by GCMS and triglycerides (TG) by spectrophotometric assay as detailed below.

4.2.3.1 Kinetic calculations

Assuming steady state conditions after 180minutes of infusion (Baar et al. 2005), the rate of appearance (Ra) of palmitate in plasma was calculated using equation 1.

Equation 1

R_a *(µmol/kg/min) = F/IE*

where F is the isotope infusion rate (µmol/kg/min) and IE is the isotopic enrichment (the tracer to tracee ratio [TTR]).

An index of the contribution of circulating FFAs to TG pools in tissues and plasma was estimated using equation 2 (Baar et al. 2005;Donnelly et al. 2005;Jung et al. 1999).

Equation 2

Fraction of TG palmitate derived from plasma FFA =
IE of $[^{13}C]_4$-palmitate in TG palmitate/ IE of $[^{13}C]_4$- palmitate in plasma

The absolute contribution of circulating palmitate to tissue or plasma TGs was calculated by correcting for the TG pool size using equation 3.

Equation 3

Absolute palmitate uptake = fraction of TG palmitate derived from plasma x TG pool size

where tissue and plasma TG pool sizes were calculated by multiplying TG concentration by tissue weight (assuming adipose tissue to be composed of pure TG of molecular weight 861 (Frayn 1983)), or estimated plasma volume (3.5% body weight (Huang et al. 2006)) respectively.

4.2.4 *De novo* lipogenesis studies

To assess DNL, in other groups of mice (n=8/group), $[^{13}C]_2$-acetate was added to diets for 5 days before sacrifice and incorporation of ^{13}C into TG assessed in liver and plasma using mass isotopomer distribution analysis (MIDA) after analysis of TG-derived palmitate and its labeled isotopomers by GCMS (Baar et al. 2005;Hellerstein and Neese 1999). Mice were housed individually and fed diets as above in a paste (225g powdered chow as above with 45ml water) packed into 50ml plastic tubes to facilitate weighing of food intake for the duration of the study. For the final 5 days, labeled $[^{13}C]_2$-acetate (4.5g) was added to the water before preparation of the paste. Tissues were collected as described above.

4.2.4.1 Mass isotopomer distribution analysis

GCMS was used to determine the pattern of incorporation of $[^{13}C]_2$-acetate into newly synthesized hepatic TG FFAs using the method of Chinkes *et al* (Chinkes et al. 1996) as described in detail in Section 2.7.2.4.

4.2.5 Hepatic triglyceride export studies

In further mice (n=7-8/group), following a 4 hour fast, hepatic VLDL export was assessed by measuring the rate of accumulation of TG (quantified by spectrophotometry) in plasma from serial tail nicks taken at 0, 30, 60, 120 and 180minutes following administration of tyloxapol (Triton W1339) by tail vein injection (500mg/kg; 15% v/v in normal saline)(Li et al. 1997). Equation 4 converted the rate of appearance per plasma volume into whole body rate of appearance, and plasma volume was estimated as 3.5% of body weight (Huang et al. 2006).

Equation 4
TG export (mg/kg/hour) =
[slope of line (mg/dl/min) x estimated plasma volume (dl)] / [weight (kg) x 60]

4.2.6 Hepatic inflammation studies

In an additional study (n=10/group), livers were fixed in 10%v/v formalin for immunohistochemical analysis after two weeks of dietary interventions, as above. In addition, fasting plasma samples from these mice were used for measurement of liver function tests and total plasma FFAs.

4.2.7 Biochemical Assays

4.2.7.1 Lipid extraction

Plasma free fatty acids were extracted and derivatised using a modified method of Patterson *et al* (Patterson et al. 1999) as described in Section 2.9.1.

To measure palmitate enrichment of plasma and hepatic TGs, lipids were extracted using a modified Folch technique (Folch et al. 1957) and the TG fraction separated by solid phase extraction (Burdge et al. 2000). TGs were then hydrolysed to their constituent FFAs and derivatised using acidified methanolic sulphuric acid (2% v/v) as described in Section 2.9.2.

4.2.7.2 Gas chromatography mass spectrometry

GC-MS conditions were as described in Section 2.9.3 and fatty acid methyl esters derived from plasma FFA and TG pools were analysed using selective ion monitoring of molecular ions with *m/z* 270, 272, 274 and 284, corresponding to [M+0], [M+2], and [M+4] isotopomers of methyl palmitate and the methyl heptadecanoate internal standard respectively. To avoid concentration dependent effects of methyl palmitate on ionization, each sample was analysed several times using a range of injection volumes as previously described (Patterson et al. 1998), and tracer to tracee ratios determined from a standard curve.

4.2.7.3 Enzymatic assays

Plasma and liver TGs were quantified enzymatically using a colorimetric assay (Infinity; ThermoTrace, Australia), as described in Section 2.10. Plasma FFAs were measured in duplicate using a commercial kit (Wako, Fuggerstrabe, Germany), and liver function tests, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST), measured using commercial colorimetric kits (Randox laboratories, Co. Antrim, UK) adapted for use on a Cobras centrifugal analyser (Roche Dignostics Ltd, Welwyn Garden City, UK) as described in Section 2.10.

4.2.8 Immunohistochemistry

Paraffin embedded sections of liver were stained using an anti-granulocyte antibody (108413 Rat anti-mouse GR1 antibody, Cambridge Biosciences, Cambridge, UK) as described in detail in Section 2.5.3.3.

4.2.9 Statistical analysis

Results are presented as mean ± standard error (SEM) and were compared by one way ANOVA, with Tukey's post hoc multiple comparison test when appropriate.

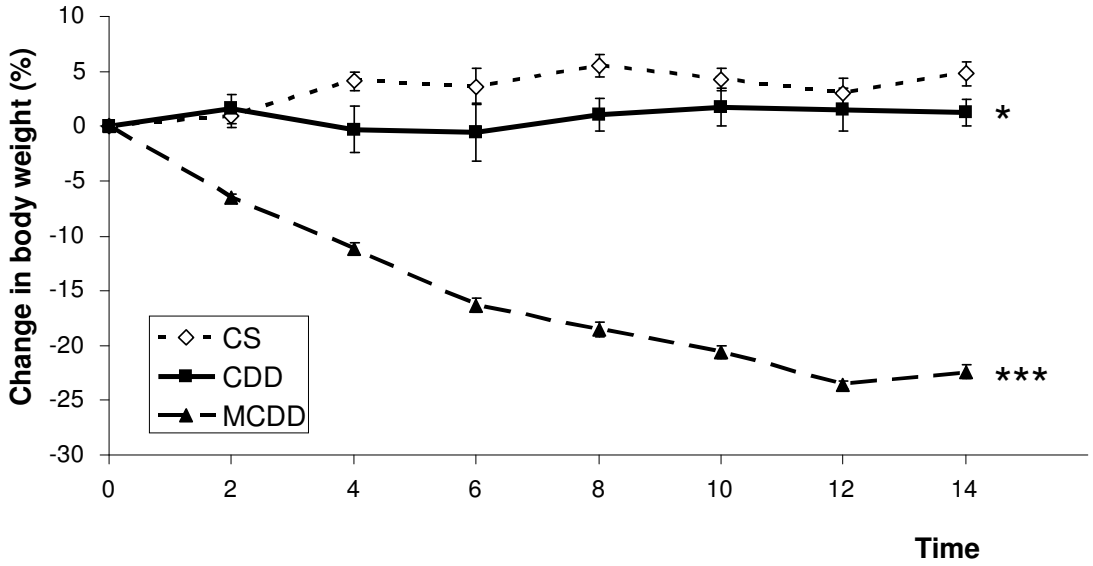
4.3 RESULTS

4.3.1 Body composition and liver triglycerides

The three groups of mice were the same weight initially. Consistent with previous studies (Rinella et al. 2008;Rizki et al. 2006) mice on MCDD lost weight (Figure 4.1), with marked loss of both subcutaneous and epididymal fat (Table 4.1). CDD mice gained weight, but less than mice on control diet (Figure 4.1). Food intake (measured in the DNL study) was reduced on MCDD compared to both control diet and CDD (Table 4.1). However, mice were otherwise active and healthy, displaying no external signs of distress.

Figure 4.1 Effect of choline ± methionine deficiency on body weight

*Data points represent mean ± SEM. N= 8-30/group depending on time point. Final weights were analysed by one away ANOVA with Tukey`s post hoc comparison test. CS, control diet; CDD, choline deficient diet; MCDD, methionine and choline deficient diet. *P<0.05, ***P<0.001.*



Liver triglyceride pool size was variable, but similarly increased in both CDD and MCDD mice compared to controls after two weeks of dietary intervention (Table 4.1).

Table 4.1 Effect of choline ± methionine deficiency on liver weights and triglycerides, adipose depot weights and food intake

Data are means ± SEM, analysed by one way ANOVA with Tukey`s post hoc tests where appropriate. $^{*}P<0.05$, $^{***}P<0.001$ vs CS; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ vs CDD. Data were collected in different experiments and combined here, hence varying numbers: $^{\$}n= 21-23$/group, $^{\S}n= 7-8$/group; all other variables n=28-30/group.

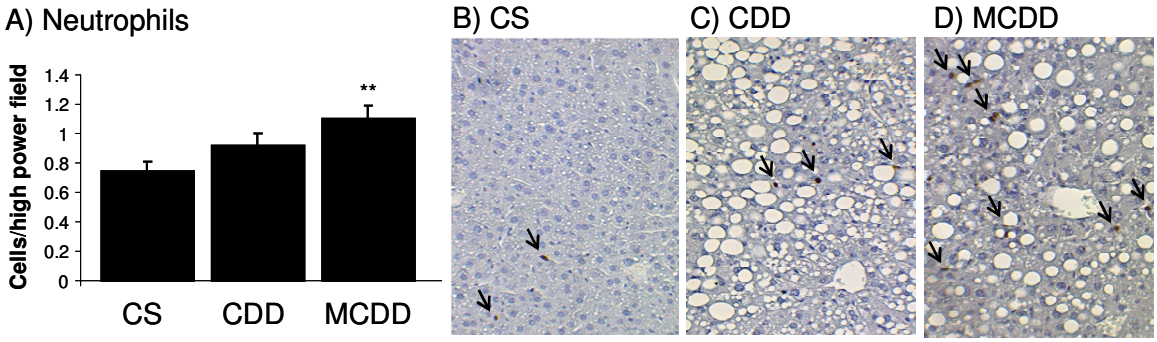
	Control diet (CS)	**Choline deficient diet (CDD)**	**Methionine and choline-deficient diet (MCDD)**
Liver weight (% body weight)	4.0±0.1	4.7±0.1***	3.8±0.1†††
Liver TG pool (µmol/liver)$^{\$}$	24.37±2.4	45.94±3.9***	43.30±3.5***
Adipose depot weights (% body weight)			
Subcutaneous$^{\$}$	1.5±0.2	1.3±0.1	0.7±0.1$^{***/\dagger\dagger}$
Epididymal$^{\$}$	1.6±0.1	1.4±0.1	0.4±0.0$^{***/\dagger\dagger\dagger}$
Mesenteric§	0.3±0.1	0.4±0.1	0.1±0.0$^{*/\dagger\dagger\dagger}$
Food intake (g/kg body weight/day)§	127.9±4.5	130.8±2.8	104±2.7$^{***/\dagger\dagger\dagger}$
Liver function tests§			
Bilirubin (µmol/l)	4.9 ± 0.8	4.5 ± 0.7	5.3 ± 0.6
ALT (U/L)	31 ± 3	48 ± 4	231 ± 79$^{\dagger/*}$
AST (U/L)	303 ± 86	224 ± 22	507 ± 90†

4.3.2 Hepatic inflammation

Steatohepatitis was confirmed in the MCDD mice at two weeks, with substantially increased plasma ALT (Table 4.1) and an inflammatory infiltrate in the liver on immunohistochemistry (Figure 4.2). In contrast, there was no elevation in plasma transaminases or liver inflammatory infiltrate in CDD mice.

Figure 4.2 Effects of choline ± methionine deficiency on liver inflammatory cell infiltration

*Data are mean ± SEM for n= 10 per group. Two weeks of a methionine and choline deficient diet (MCDD) but not choline deficient diet (CDD) produced an increase in neutrophil staining. A) Average cell counts of anti GR1 positive staining cells per high power fields, *p<0.05. Exemplary GR1 positive staining cells in B) controls group (CS), C) CDD and D) MCDD. Original magnification 250x.*



4.3.3 Fatty acid flux

Despite the loss of peripheral fat depots in the MCDD group, the rate of appearance (Ra) of palmitate did not differ between the three groups (Table 4.2). In keeping with this, fasting FFAs were not statistically different: 670±62 vs 602±91 vs 887±220 µmol/l for CS, CDD, and MCDD respectively.

The contribution of FFAs to triglyceride pools was assessed in liver and plasma. Despite marked differences in the fractional contribution of plasma palmitate to liver triglycerides, there was no effect of diet on the absolute contribution of plasma

palmitate to the liver triglyceride-derived palmitate pool. However, the contribution of plasma palmitate to circulating triglyceride-palmitate was reduced in MCDD mice.

Table 4.2 Effect of choline ± methionine deficiency on fatty acid flux measured by dilution of $[^{13}C]_4$ palmitate tracer

Data are means ± SEM, analysed by one way ANOVA with Tukey`s post hoc tests where appropriate: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs CS; $^{\dagger}P<0.05$ vs CDD. N= 12-15/group.

	Control diet (CS)	**Choline deficient diet (CDD)**	**Methionine and choline-deficient diet (MCDD)**
Ra palmitate (µmol/kg/min)	27.16±3.46	26.25±2.27	28.34±3.46
Fractional contribution to liver TG pool (%)	34.03±5.11	11.38±1.95***	14.56±1.93***
Liver TG pool (µmol/liver)	23.98±3.69	51.27±6.15**	40.52±4.58*
Absolute contribution to liver TG pool (µmol/liver)	6.88±1.12	5.10±0.87	5.83±0.93
Fractional contribution to plasma TG pool (%)	39.55±5.25	28.80±4.30	23.77±5.23
Plasma TG pool size (µmoles)	0.67±0.05	0.67±0.06	0.46±0.3*/†
Absolute contribution to plasma TG pool (µmoles)	0.26±0.04	0.19±0.04	0.11±0.03*

4.3.4 *De novo* lipogenesis

MIDA of triglyceride palmitate following ingestion of $[^{13}C]_2$-acetate is summarized in Table 4.3. There were no differences in fractional synthesis rates, i.e. the percentage of newly synthesized fatty acids contributing to the triglyceride pool, between diets. However, taking into account the differing hepatic triglyceride pool sizes, there was a marked increase in absolute rates of hepatic DNL in MCDD mice, which was not detected in CDD mice. Despite increased hepatic DNL in MCDD mice, a smaller amount of newly synthesized fatty acids appeared in the plasma triglyceride pool.

Table 4.3 Effect of choline ± methionine deficiency on hepatic de novo lipogenesis and newly synthesised hepatic fatty acid export

Absolute synthesis rates were calculated from mass isotopomer distribution analysis of triglyceride derived palmitate following dietary [$^{13}C]_2$-acetate labeling. The absolute synthesis rate of plasma TGs represents the hepatic export of newly synthesised fatty acids. Results are means ± SEM, analysed by one way ANOVA with Tukey's post hoc tests where appropriate. $^{*}P<0.05$, $^{**}P<0.01$ vs CS. N=8/group, except plasma TG data, where n=5-7/group

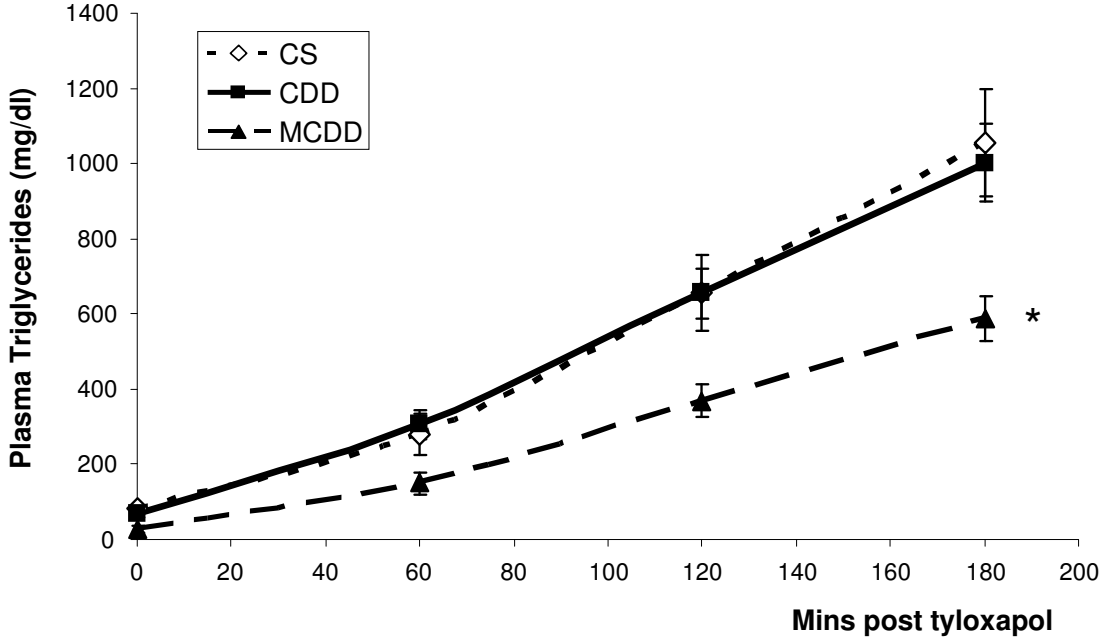
	Control diet (CS)	Choline deficient diet (CDD)	Methionine and choline-deficient diet (MCDD)
Liver			
Acetyl CoA enrichment (%)	7.6±0.6	8.9±0.5	7.2±0.3
Liver TG pool (µmol/liver)	25.0±2.3	38.2±4.0	48.5±5.0**
M+2 Enrichment	0.104±0.009	0.121±0.008	0.132±0.007
M+4 Enrichment	0.029±0.003	0.041±0.003*	0.036±0.003
Fractional synthesis rate (%/day)	5.6±0.7	5.9±0.4	6.8±0.3
Absolute synthesis rate (µmol/day)	1.4±0.3	2.3±0.4	3.4±0.4**
Plasma TG			
Acetyl CoA enrichment (%)	7.6±0.5	8.9±0.4	7.4±0.4
TG pool (µmoles)	1.7±0.3	1.5±0.2	1.0±0.1*
Fractional synthesis rate (%/day)	6.5±0.5	6.2±0.4	6.6±0.7
Absolute synthesis rate (µmol/day)	0.33±0.05	0.27±0.04	0.19±0.03*

4.3.5 Triglyceride export

Intravenous tyloxapol induced a linear increase in plasma triglyceride levels, which was not altered in CDD mice but was markedly reduced in MCDD mice (Figure 4.3).

Figure 4.3 Effect of choline ± methionine deficiency on plasma triglycerides (TG) following intravenous tyloxapol

*Hepatic TG export was reduced in the MCDD group only. Data are expressed as mean ± SEM. Rate of TG export (142±21 vs 122±15 vs 80±7mg/kg/hr for CS, CDD and MCDD respectively) was calculated from the linear portion of the graphs using Equation 6 and analysed by one away ANOVA with Tukey`s post hoc comparison test where appropriate. N=7-8/group. *P<0.05 vs CS.*



4.4 DISCUSSION

These data reveal marked differences in fatty acid metabolism in mice fed a methionine and choline-deficient diet (MCDD) versus a simple choline-deficient diet (CDD), which may promote the development of hepatocyte damage and steatohepatitis in the MCDD but not CDD model at similar levels of total liver

triglyceride accumulation. Measurement of plasma triglycerides after tyloxapol confirmed impaired hepatic triglyceride export in the MCDD mice only, with palmitate and acetate tracer administration demonstrating that export of both newly synthesized and plasma derived re-esterified FFAs is impaired. Reduced liver secretion of fatty acids (in VLDL) and hence supply to adipose tissue, rather than increased lipolysis and adipose release of fatty acids, may therefore contribute to loss of body fat in MCDD mice. In addition, hepatic *de novo* lipogenesis (DNL) is increased in MCDD mice, potentially exacerbating accumulation of fatty acids as well as triglycerides in hepatocytes. In contrast, in choline deficiency without methionine deficiency (CDD) there were no significant changes in liver triglyceride secretion, fatty acid turnover or DNL. No increased fatty acid esterification was measured in CDD mice by $[^{13}C]_4$ dilution, as previously hypothesized (Raubenheimer et al. 2006), although we cannot exclude that a co-existing intrahepatic fatty acid pool exists that is not successfully labeled in our tracer studies and contributes to triglyceride accrual.

Methionine and choline are both key nutrients necessary for the synthesis of phosphatidyl choline and export of VLDL from the liver (Li and Vance 2008;Zeisel and Blusztajn 1994). It has been widely assumed that hepatic triglyceride accumulation in both CDD and MCDD is the result of impaired VLDL export (reviewed in (Kulinski et al. 2004)). Impaired VLDL export has recently been confirmed using tyloxapol in MCDD mice (Rinella et al. 2008). However, in CDD mice this explanation is based on an experiment which demonstrated reduced incorporation of radiolabeled palmitate tracer into plasma triglycerides and retention of radioactivity in the liver in rats acutely (15 hours) after introducing a CDD (Lombardi et al. 1968). These observations could be explained by increased dilution of tracer in the expanded triglyceride storage pool in liver from which VLDL is exported, or increased mitochondrial oxidation of palmitate and release of radioactivity. Here, we show that CDD mice studied after 2 weeks of dietary manipulation do not share an impairment of hepatic VLDL export with MCDD mice. Not only was triglyceride accumulation following tyloxapol normal in CDD mice, but incorporation of palmitate tracer into plasma triglycerides was intact following

tracer palmitate or acetate administration. This conclusion is supported by recent *in vitro* studies in murine liver cell lines (Kulinski et al. 2004).

The difference in VLDL export between CDD and MCDD alone is unlikely to explain the contrasting risk of steatohepatitis. Both apolipoprotein B100 (apoB100) and the Mttp encoded microsomal triglyceride transfer protein are essential for hepatic triglyceride export, and deficiency of these proteins in both mice and humans leads to hepatic steatosis (Bjorkegren et al. 2002;Li et al. 1997;Raabe et al. 1999;Schonfeld et al. 2003;Wetterau et al. 1992), but critically is not associated with the inflammation seen in the MCDD model. However, impaired VLDL export may sensitize the liver to additional toxic insults, as evidenced by an increase in lipopolysaccharide induced inflammation in mice with reduced hepatic *Mttp* expression (Charlton et al. 2002). Moreover, limited circumstantial evidence suggests that patients with steatohepatitis have impaired apoB-dependent triglyceride export (Tietge et al. 1999) and more frequent polymorphisms in the *Mttp* gene (Schonfeld et al. 2008).

The impaired hepatic triglyceride export and steatosis found in the ApoB-38.9 mouse model of familial hypobetalipoproteinaemia is associated with suppression of *de novo* lipogenesis, perhaps reflecting an adaptive mechanism (Lin et al. 2002). Using deuterium as a tracer, DNL has previously been shown to be lower in C3H/HeOuJ mice fed a high sucrose, lipogenic MCDD than in mice fed a methionine/choline supplemented lipogenic diet; however, in keeping with our findings, absolute DNL rates were still markedly elevated on the lipogenic MCDD diet compared to standard chow fed controls (Rizki et al. 2006). DNL has not previously been investigated in CDD mice using *in vivo* tracer techniques. We found that DNL is markedly increased in MCDD mice but not in CDD mice, although this result should be interpreted with the caveat that the ASR calculation is dependent on the hepatic triglyceride pool size which, surprisingly, was not significantly increased in the CDD group in the *de novo* lipogenesis experiment (Table 4.3). The difference in DNL between CDD and MCDD mice was also not statistically significant. We opted to use a doubly labeled acetate tracer to improve accuracy of GCMS for MIDA as suggested by Chinkes *et*

al (Chinkes et al. 1996), although due to theoretical concerns over intrahepatic acetyl CoA gradients, the use of an acetate tracer may have slightly underestimated DNL (Bederman et al. 2004).

It does not appear that alterations in fatty acid supply to the liver from peripheral tissues is important in the MCDD or CDD model. We studied mice after two weeks of dietary manipulation when the fatty liver phenotypes are established. Weight loss in the MCDD mice was rapid initially and slowing by two weeks (Figure 4.1). It is therefore possible that we have under-estimated any acute increase in peripheral fatty acid release. However, increased peripheral fat turnover does not appear necessary to maintain steatohepatitis. There is also debate as to whether the Ra of palmitate should be expressed relative to fat mass, fat free mass or total body weight (Koutsari and Jensen 2006). We expressed the Ra of palmitate per unit of body mass to best represent the total flux of fatty acids to the liver, although clearly the Ra of palmitate would be higher in the MCDD group if expressed relative to fat mass.

We have not made *in vivo* measurements of hepatic fatty acid oxidation. These are difficult, particularly in mice. Even if we had collected expired carbon dioxide or conducted indirect calorimetry (Baar et al. 2005), this would not have been specific to hepatic mitochondrial oxidation. Whole body β-oxidation rates have been shown to be elevated in MCDD mice, with an associated increase in CPT1a activity and palmitate oxidation measured *ex vivo* (Rizki et al. 2006), although this was not sufficient to increase plasma β–hydroxybutyrate levels (Rinella et al. 2008) or to compensate for the increased hepatic fatty acid load secondary to impaired VLDL export and increased DNL. There is accruing evidence that fatty acid oxidation may be less efficient in the MCDD model (Romestaing et al. 2008), leading to increased microsomal and peroxisomal oxidation, generating reactive oxygen species and lipid peroxides thought to be important in the pathogenesis of inflammation and liver cell necrosis (Leclercq et al. 2000). Clearly the impact in the liver of increased FFA generation and impaired export, as we have described here, would be exacerbated by any relative failure of fatty acid oxidation (Ghoshal and Farber 1993). Another technical limitation is that repeated sampling is not possible in mice to confirm that

steady state of tracer enrichments has been achieved. This may be most relevant to the incorporation of labelled palmitate within triglyceride pools, although even if steady state has not been achieved then the index we used in equation 2 is likely to reliably reflect the rate of change. Finally, it would be ideal to conduct pair-feeding experiments to match weight loss in CDD and MCDD mice, although such is the severity of weight loss in MCDD mice this would be technically very challenging.

In summary, using a number of methodologies, including *in vivo* tracer techniques, we have demonstrated important differences in fatty acid metabolism which associate with contrasting susceptibility to steatohepatitis in CDD and MCDD mice. We speculate that the combination of abnormalities in fatty acid metabolism in MCDD mice leads to accumulation of free fatty acids within the liver that is disproportionate to the accumulation of triglycerides and predisposes MCDD mice to complications. It will be important to establish if similar changes in fatty acid metabolism are predictive of steatohepatitis and liver damage in other circumstances, including in humans.

5 Chapter 5-Hepatic glucocorticoid metabolism in choline ± methionine deficient models of non-alcoholic fatty liver disease and carbon tetrachloride induced liver fibrosis

5.1 INTRODUCTION

In Chapter 3 I examined the susceptibility of two contrasting models of fatty liver to carbon tetrachloride (CCl_4) induced hepatic fibrosis. I found that steatohepatitis induced by a methionine and choline deficient diet (MCDD) did not increase susceptibility to fibrosis compared to isolated steatosis induced by a methionine supplemented choline deficient diet (CDD). In this chapter I investigate whether changes in key enzymes regulating hepatic glucocorticoid levels, predicted to reduce hepatic glucocorticoid levels, contribute to the development of steatosis, NASH, or progression to liver fibrosis.

There is accruing evidence that tissue-specific dysregulation of glucocorticoid metabolism contributes to the pathogenesis of the *metabolic syndrome* incorporating central obesity, type 2 diabetes, dyslipidaemia, and hypertension (Seckl and Walker 2001;Tomlinson et al. 2004). Non-alcoholic fatty liver disease (NAFLD) is considered to be the hepatic manifestation of the metabolic syndrome (Marchesini et al. 2001), which may be of particular relevance as the liver plays a central role in glucocorticoid hormone metabolism (Stimson et al. 2009). Glucocorticoids have both wide-ranging metabolic (Andrews and Walker 1999) and immunomodulatory (Chapman et al. 2009) properties, and a reduction in local glucocorticoid levels, either via increased inactivation or impaired regeneration, could have significant consequences for both the development and progression of NAFLD from simple steatosis to steatohepatitis, fibrosis and/or cirrhosis e.g. through effects on intrahepatic fatty acid metabolism or pro-inflammatory/pro-fibrotic cytokine production (Gentile and Pagliassotti 2008;Syn et al. 2009;Yamaguchi et al. 2007).

Corticosterone, the principle glucocorticoid in rodents, can be generated from its inactive metabolite 11-dehydrocorticosterone by 11β-HSD1 in the liver, amplifying glucocorticoid levels and receptor activation within tissues (Seckl and Walker 2001;Tomlinson et al. 2004). Conversely, glucocorticoids are inactivated by the hepatic A-ring reductases (5α-reductase type 1 and 5β-reductase) and 3α-hydroxysteroid dehydrogenase (3α-HSD) (Tomlinson et al. 2004) (see Figure 1.4 in Chapter 1).

Dysregulation of one or more of these enzymes, thereby altering local glucocorticoid levels, can influence the development of NAFLD (and insulin resistance) e.g. transgenic mice overexpressing 11β-HSD1, in either adipose tissue (Masuzaki et al. 2001) or the liver (Paterson et al. 2004), have increased hepatic triglycerides. Conversely, in the majority of rodent models with fatty liver, hepatic glucocorticoid signalling is abnormal with suppressed 11β-HSD1 and increased A-ring reductase activity, predicted to lower intrahepatic glucocorticoid levels (Livingstone et al. 2005;Livingstone et al. 2009a). It is unclear whether abnormalities of glucocorticoid metabolism also influence hepatic inflammation as few rodent models of NAFLD spontaneously develop steatohepatitis and human studies are problematic. The influence of dysregulation of glucocorticoid metabolism on the progression to hepatic fibrosis is also unknown.

I have compared hepatic glucocorticoid metabolism in CDD and MCDD dietary models of steatosis and steatohepatitis respectively, including following induction of fibrosis with CCl_4, to investigate the contribution of abnormalities of glucocorticoid signalling to progressive liver disease in NAFLD.

5.2 RESEARCH DESIGN AND METHODS

Animals and procedures

All experiments were carried out under a UK Home Office animal license using 12 week old male C57BL/6J mice (Harlan, Bicester, UK) maintained under controlled

conditions of light and temperature. Following a 2 week period of acclimatisation, mice (n=7/group) were offered either control (choline and methionine supplemented) chow (CS), choline deficient diet (CDD) or methionine-choline deficient diet (MCDD) (Dyets Inc., Bethlehem, PA) for 4 weeks and fibrosis induced with twice weekly intraperitoneal injections of 0.3µl/g CCl_4 (1:3 v/v in olive oil). Controls were injected with olive oil alone. Mice were culled 3 days following the last CCl_4 injection and sections of liver snap frozen and stored at -80ºC prior to analysis.

5.2.1 Messenger RNA (mRNA) extraction and quantification

mRNA transcripts were quantified using real time PCR as described in Section 2.6, with primers and probes designed from the Roche UPL library (see Table 5.1). The abundance of transcript levels were normalised to mean values of internal control house keeping genes (GAPDH; Assay ID Mm99999915_gl, and cyclophilin A; Assay ID Mm02342429_g1, Applied Biosystems, Warrington, UK; TBP, Roche, Burgess Hill, UK).

Table 5.1. Primers sequences and probes

mRNA of Gene	Genbank RefSeq	Sequence	Direction	UPL probe no.
Hsd11b1 (11β-HSD1)	NM_008288.2	tctacaaatgaagagttcagaccag gccccagtgacaatcacttt	Forward Reverse	1
Srd5a1 (5α-reductase1)	NM_175283.3	gggaaactggatacaaaataccc gcacgagctccccaaaata	Forward Reverse	41
Akr1d1 (5β-reductase)	NM_145364.2	gaaaagatagcagaagggaaggt gggacatgctctgtattccataa	Forward Reverse	103
Akr1c6 (3α-HSD)	NM_030611.3	tggggtgtccacctttaacc tccctgattgagataaggatgac	Forward Reverse	78
Tbp	NM_013684.3	ggcggtttggctaggttt gggttatcttcacacaccatga	Forward Reverse	107

5.2.2 In vitro enzyme activity assays

In vivo, 11β-HSD1 acts as a keto-reductase to convert inactive 11-dehydrocorticosterone to corticosterone. In contrast, *in vitro*, dehydrogenation predominates and the assay is therefore measured in this direction, ensuring an adequate supply of nicotinamide adenine dinucleotide phosphate ($NADP^+$) cofactor. 5β-Reductase converts both corticosterone and 11-dehydrocorticosterone to 5β metabolites, and its *in vitro* activity is assessed by a two step conversion of corticosterone to 5β-tetrahydrocorticosterone, also involving 3α-HSD, in which 5β-reductase is the rate limiting step (Livingstone et al. 2000a). It is not possible to undertake an enzyme activity assay of 5α-reductase as is unstable *in vitro* (Eicheler et al. 1995), and the activity of 3αHSD was not determined as it is not rate limiting (Barat et al. 2007).

100mg of liver was homogenised in 1ml of KREBS buffer in 2ml eppendorfs and centrifuged at 1000 x g for 10mins at 4ºC. The supernatant was then resuspended in KREBS buffer and the protein content determined as follows using a colourimetric assay. Protein standards were prepared from a 10mg/ml bovine serum albumin solution (Promega, Madison, WI, USA) diluted in KREBS buffer to give a series of concentrations ranging from 0-1.2mg/ml. Protein concentration was then determined using a Bio-Rad protein assay solution (Bio-Rad Laboratories GmbH, Munich, Germany) diluted 1 in 5 in distilled water. 10µl of the tissue homogenate or protein standard was added to 190µl of the diluted assay solution and samples run in duplicate in 96 well plates (BD Falcon, Becton Dickinson, Franklin Lakes, NJ, USA). After incubating for 30mins at room temperature, absorbance was measured at 590nm on an Optimax microplate reader (Molecular Devices Ltd, Berkshire, UK) and the mean of each duplicate calculated, accepting a variability of <10%. The standard curve (prepared from the protein standard dilutions) was deemed acceptable if the R^2 value was >0.99. Samples were frozen at -20ºC prior to the activity assay.

5.2.2.1 11β-HSD1 activity assay

After thawing at room temperature, samples were diluted in KREBS buffer to normalise protein concentrations. Tissue homogenates (40µg/ml) were incubated in duplicate at 37ºC in KREBS buffer containing 0.2% w/v glucose, NADP (2mmol/l), 1,2,6,7-[^{3}H]$_4$-corticosterone (10nmol/l), and unlabelled corticosterone (1.99µmol/l) in a final reaction volume of 500µl. Negative controls were also run without either corticosterone, or NADP, or protein. Protein concentrations and incubation periods were chosen to achieve first order kinetics.

A 200µl aliquot of the reaction mixture was removed after 60mins incubation and the reaction halted and steroids extracted by the addition of 2ml ethyl acetate. After mixing, the organic phase was transferred into borosilicate glass reaction tubes and evaporated under oxygen free nitrogen at 60ºC. Samples were redissolved in 1ml of mobile phase (water, acetonitrile, and methanol; 65:15:25), transferred to clear shell HPLC vials (Supelco, Poole, Dorset, UK), and stored at -20ºC prior to high performance liquid chromatography (HPLC).

Steroids were separated by HPLC comprising a Waters auto-injector, mobile-phase pump, C18 reverse phase Sunfire 5µm (150mm length, 4.6mm i.d.) column (Hertfordshire, UK), column heater, and radioactivity monitor linked to a scintillation fluid pump (Berthold, Leeds, UK). The mobile phase flow rate was 1.5ml/min, and the scintillant (Gold multipurpose flow scintillation cocktail, Meridian, Surrey, UK) flow rate was 2ml/min. The β-emissions from the tritiated steroids cause the scintillation, producing photoemissions measured by the photodiode.

The column heater was set to 40ºC and radioactive standards were injected at the start of every run to confirm the identity and retention time of the peaks. The retention times for 11-dehydrocorticosterone and corticosterone were approximately 14mins and 19mins respectively. Chromeleon software version 6.5 (Dionex, Surrey, UK) controlled the system and was used to integrate the areas under the peaks. 11β-HSD1 velocity was calculated from the conversion of 1,2,6,7-[^{3}H]$_4$-corticosterone to

1,2,6,7-[^{3}H]$_4$-11-dehydrocorticosterone using the formula: area under the peak (AUP) [^{3}H]$_4$-dehydrocorticosterone / (AUP [^{3}H]$_4$-corticosterone + AUP [^{3}H]$_4$-dehydrocorticosterone). Results were reported as pmol/hour/mg protein (correcting for the total concentration of corticosterone in the reaction mixture).

5.2.2.2 5β-Reductase activity assay

This assay was carried out on the cytosolic fraction obtained after ultracentrifugation of the tissue homogenates prepared as described above. After centrifuging the homogenate at 1000 x g for 10mins at 4ºC, the supernatant was centrifuged at 14,000 x g for 20mins. The cytosolic fraction was obtained by centrifugation of the resultant supernatant at 100,000 x g for 60mins (leaving a microsomal pellet), and the protein concentration was determined as before.

Cytosolic samples were diluted in assay buffer (40mmol/l Na_2HPO_4, 320mmol/l sucrose, and 1mmol/l dithiothreitol, pH 7.5) to normalise protein concentrations to 100µg in 180µl. A 250µl reaction mixture was prepared with the addition of 50µl co-factor mix (containing 10mmol/l NADPH, 25mmol/l glucose-6-phosphate, and 5units/ml glucose-6-phosphate dehydrogenase), plus 20µl substrate mix (containing 1250nmol/l 1,2,6,7-[^{3}H]$_4$-corticosterone and 123.75µmol/l corticosterone). This gave final concentrations of 2mmol/l NADPH, 5mmol/l glucose-6-phopshate, 1unit/ml glucose-6-phosphate dehydrogenase, 100nmol/l 1,2,6,7-[^{3}H]$_4$-corticosterone and 9.975µmol/l corticosterone. The assay was performed in glass reaction tubes and after briefly vortexing, samples were incubated at 37ºC for 16hours. Control samples were also run containing either no cytosol or no cofactor mix. The reaction was stopped by the addition of 2.5ml ethyl acetate and tubes vortexed. Samples were stored overnight at -20ºC.

The halted reaction mixtures were centrifuged at 1000 x g for 5mins at 4ºC and the upper organic phase transferred to fresh glass tubes and dried down under oxygen free nitrogen at 60ºC. Samples were redissolved in 200µl of HPLC mobile phase (water, acetonitrile, and methanol; 65:15:25) and transferred to clear shell HPLC vials (Supelco, Poole, Dorset, UK).

Steroids were separated using the same HPLC system, column and mobile phase as above, heated to 35ºC, and quantified by on-line scintillation counting. The mobile phase was run at 1ml/min for a run time of 45mins. 1,2,6,7-[^{3}H]$_4$-corticosterone and 1,2,6,7-[^{3}H]$_4$-5β-tetrahydrocorticosterone had retention times of ~25 and 36mins respectively. 5β-Reductase activity was assessed by conversion of 1,2,6,7-[^{3}H]$_4$-corticosterone to 1,2,6,7-[^{3}H]$_4$-5β-tetrahydrocorticosterone.

5.2.3 Statistical analysis

Results are presented as mean ± standard error (SEM). Between group differences were compared by one way ANOVA, assuming equal variances, with Tukey's post hoc multiple comparison test when appropriate. An overall effect of CCl_4 treatment was determined by two way ANOVA and the effect in each dietary group assessed by Student`s t-test (or the non-parametric equivalent) using Graphpad Prism software (La Jolla, CA, USA) and SPSS (IBM, NY, USA).

5.3 RESULTS

5.3.1 Effect of choline ± methionine deficiency on glucocorticoid signalling

There was no difference in abundance of transcripts of 11β-HSD1, 5α-reductase, 5β-reductase, or 3α-HSD between CDD mice with simple steatosis and controls fed a supplemented diet (Figure 5.1). In contrast, MCDD mice had reduced 11β-HSD1 and 5α-reductase 1 mRNA levels but no change in 3α-HSD or 5β-reductase transcript abundance. Results of enzyme activity assays were somewhat different: again, there was no abnormality in CDD mice, but MCDD mice exhibited no significant difference in 11β-HSD1 or 5β-reductase activities (Figure 5.2).

5.3.2 Effect of carbon tetrachloride on glucocorticoid metabolism in control mice fed a methionine and choline supplemented (CS) diet

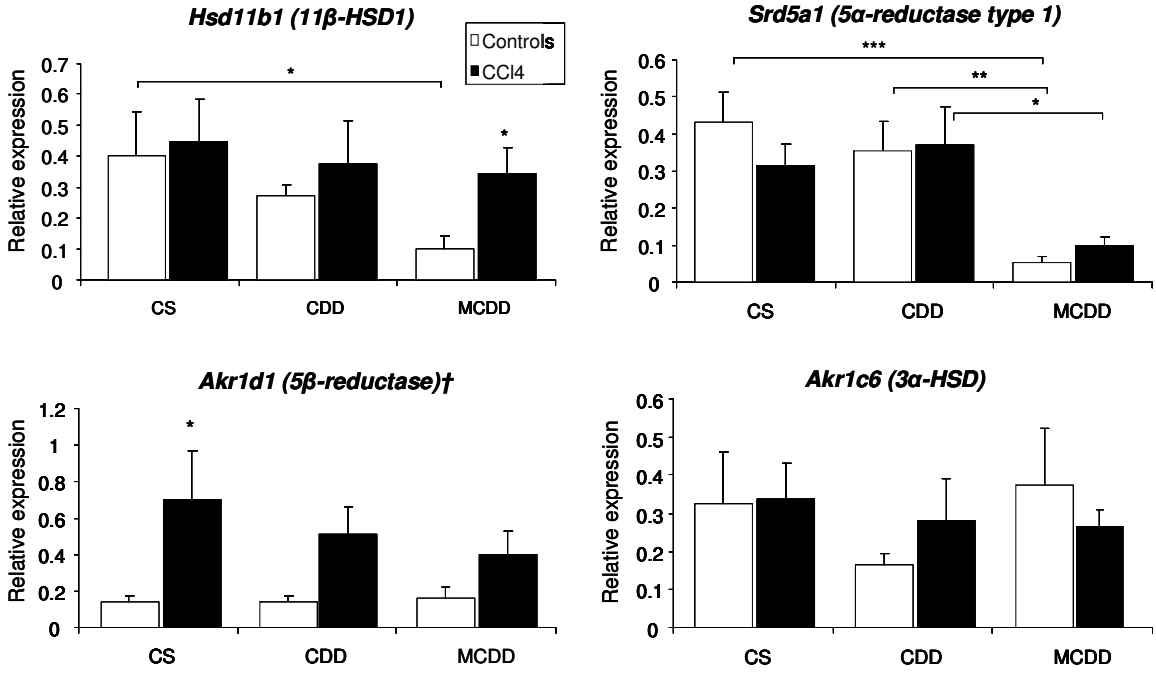
There was no change in 11β-HSD1 mRNA levels or activity in response to CCl_4 in CS fed mice (Figures 5.1 and 5.2). Similarly, CCl_4 did not influence mRNA levels of 5α-reductase or 3α-HSD, and although there was an increase in abundance of 5β-reductase transcripts, this was not confirmed by enzyme activity assay.

5.3.3 Effect of carbon tetrachloride on glucocorticoid metabolism in methionine ± choline deficiency

CCl_4 had no effect on mRNA levels of 11β-HSD1, 5α-reductase and 3α-HSD (Figure 5.1) or 11β-HSD1 enzyme activity in CDD mice (Figure 5.2). Similarly, in MCDD mice, CCl_4 had no influence on mRNA levels of 3α-HSD and 5α-reductase, with 5α-reductase mRNA levels remaining suppressed. In contrast, 11β-HSD1 mRNA levels were no longer suppressed in CCl_4 treated MCDD mice, although this was not confimed by enzyme activity assay (Figure 5.2). CCl_4 increased 5β-reductase gene expression (Figure 5.1, $P<0.01$ by two way ANOVA), although again enzyme activity assays were not confirmatory (Figure 5.2), and indeed, 5β-reductase enzyme velocity was reduced in CCl_4 treated MCDD mice.

Figure 5.1 Hepatic gene expression studies

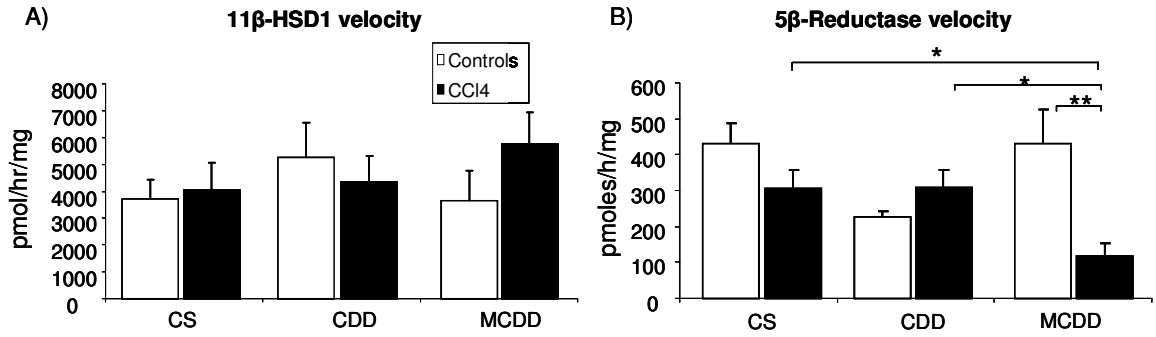
*Data are expressed as a ratio to the abundance of transcripts of control genes, as means ± SEM.. Differences between dietary groups were analysed by one way ANOVA with Tukey's post hoc comparison test where appropriate. An overall effect of CCl_4 was assessed using a two way ANOVA, with the effect in each dietary group assessed by Student's t-test (or non-parametric equivalent if appropriate). N=5-6/group. $^{*}P<0.05$, $^{**}P<0.01$, ***P<0.001*



†P<0.01 for an effect of CCl4 by two way ANOVA.

Figure 5.2 Hepatic enzyme velocities

Hepatic A) 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and B) 5β-reductase velocity determined by HPLC in choline ± methionine deficiency and following carbon tetrachloride (CCl_4) induced hepatic fibrosis. Differences between dietary groups were analysed by one way ANOVA with Tukey's post hoc comparison test where appropriate. Effects of CCl_4 treatment was analysed by Student's t-test (or non-parametric equivalent if appropriate). N=5-6/group. $^{*}P<0.05$, $^{**}P<0.01$.



5.4 DISCUSSION

These results suggest significant differences in hepatic glucocorticoid metabolism in livers with steatohepatitis secondary to a methionine and choline deficient diet (MCDD) compared to both steatotic livers (without inflammation), due to an isolated choline deficienct diet (CDD), and non-steatotic livers from mice fed a methionine and choline supplemented (CS) diet. Furthermore, despite similar hepatic triglyceride accumulation (as documented in Section 3.3.4), there were model specific differences in response to carbon tetrachloride (CCl_4) suggesting that changes in hepatic glucocorticoid signalling may play an important role in modulating their contrasting susceptibility to fibrosis.

The hepatic gene expression studies, summarised in Table 5.2, predict no change in hepatic glucocorticoid levels in the CDD model, whereas the reduced 11β-HSD1 mRNA levels in the inflamed MCDD livers predict lower intrahepatic glucocorticoids in this model, potentially contributing to the pathogenesis of

steatohepatitis. However, these results need to be interpreted with some caution given the conflicting results of the 11β-HSD1 activity assay. Furthermore, any reduction in glucocorticoid levels in MCDD mice may be offset by reduced clearance by 5α-reductase.

CCl_4 injury increased 5β-reductase transcript levels in all three dietary groups, predicted to enhance glucocorticoid clearance. However, activity assays were not supportive of these findings. Indeed, 5β-reductase enzyme velocity was specifically reduced in MCDD mice treated with CCl_4. In addition, sustained downregulation of 5α-reductase and the relative lack of downregulation of 11β-HSD1 mRNA levels in CCl_4 treated MCDD mice, would also suggest that a reduction in glucocortoid clearance, combined with a relative increase in glucocorticoid regeneration could have contributed to the reduction in fibrosis in these mice. However, enzyme activity assay findings were again conflicting.

Table 5.2 Summary of effects of choline ± methionine deficiency and carbon tetrachloride on mRNA levels of gene regulating hepatic glucocorticoid levels

	Baseline		Post CCl_4	
	CDD	MCDD	CDD	MCDD
11β-HSD1	↔	↓	↔	↑
5α-Reductase	↔	↓	↔	↔
5β-Reductase	↔	↔	↑	↑
3α-HSD	↔	↔	↔	↔

Given the opposing effects of 11β-HSD1 and 5α-reductase in MCDD mice and the discrepancies between the gene expression and enzyme activity results it is difficult to draw definitive conclusions from these results. Reasons for these differences in mRNA levels and measured enzyme velocities are unclear, but may relate to differential stability of mRNA transcripts and protein (Eicheler et al. 1995), or post-tranlational modification of proteins (Oppermann et al. 1995). Nevertheless, the current observations provide encouragement that further investigation of glucocorticoid metabolism in NAFLD will be productive.

The liver is the principle site in the body for metabolism of glucocorticoids, principally by the A-ring reductases and 3α-HSD. In addition, 11β-HSD1 is highly expressed in the liver, acting in a tissue specific manner to amplify local glucocorticoid levels. Most (Drake et al. 2005;Liu et al. 2003;Livingstone et al. 2009a;Livingstone et al. 2000a), but not all (Liu et al. 2005), studies of rodents with fatty liver, although crucially not with steatohepatitis, have found reduced hepatic 11β-HSD1 activity. Furthermore, glucocorticoid inactivating A-ring reductase activity is also often increased, possibly in response to increased glucocorticoid regeneration within adipose tissue (Liu et al. 2003;Livingstone et al. 2009a;Livingstone et al. 2000a). The lack of inflammation in these NAFLD models suggest that the changes in glucocorticoid metabolism are not the primary event inducing hepatic inflammation, but may represent a protective mechanism against the metabolic complications of obesity, whilst increasing susceptibility to factors promoting steatohepatitis.

It is unclear what is driving changes in hepatic glucocorticoid metabolism in MCDD mice, in whom, unlike other models of NAFLD, weight loss rather than obesity is a key feature. 11β-HSD1 is regulated by numerous factors, including glucocorticoids, insulin, sex steroids, IGF-1, and cytokines (Seckl and Walker 2001), although some of these factors may have opposing effects e.g. TNFα and insulin (Handoko et al. 2000), and have tissue specific effects e.g. cytokines don`t appear to upregulate hepatic 11β-HSD1 (Escher et al. 1997;Tomlinson et al. 2001). Weight loss is another potential regulator of 11β-HSD1, although recent evidence suggests this relates to dietary macronutrient content (Stimson et al. 2007), which was identical in our groups, whilst reduced leptin levels in MCDD mice are unlikely to be relevant (Livingstone et al. 2009a). Fatty acids may also be important, increasing adipose 11β-HSD1 activity *in vivo* in humans (Wake et al. 2006), although effects in hepatocytes are unknown. In contrast, factors regulating the A-ring reductases are less well studied (Livingstone et al. 2009a), and the reduced expression of 5α-reductase in the MCDD mice is unusual amongst models of NAFLD, the actions of which would oppose those of 11β-HSD1. In addition, the CDD model appears

unique in that there are no apparent changes in hepatic glucocorticoid metabolism, although this did not appear to provide any apparent benefit in reducing the development of liver fibrosis.

The "two hit" hypothesis suggests that a toxic insult, e.g. oxidative stress secondary to fatty acid lipotoxicity, is required in addition to steatosis to promote the development of hepatic inflammation (Day and James 1998). There are few easily employed models of steatohepatitis in rodents (Anstee and Goldin 2006), hence we have used a MCDD in which abnormalities of hepatic fatty acid metabolism, impaired mitochondrial beta oxidation and increased reactive oxygen species may contribute to the development of steatohepatitis (Ip et al. 2003;Kashireddy and Rao 2004;Larter et al. 2008a). However, these factors are not mutually exclusive and changes in intrahepatic glucocorticoid levels may modulate their potency e.g. glucocorticoids can reduce reactive oxygen species generation, increase hepatic antioxidant defences (Eken et al. 2006;Gil et al. 1997;Ozturk et al. 2006) and inhibit hepatocyte apoptosis, possibly via effects on NFκB signalling (Evans-Storms and Cidlowski 2000;Iida et al. 1998). It is also difficult to separate the metabolic from the anti-inflammatory effects of glucocorticoids, with glucocorticoid receptor blockade (using RU486) increasing CCl_4 induced hepatic inflammation, whilst also reducing triglyceride accumulation (Swain et al. 1999).

The effects of glucocorticoids on hepatic fibrosis have not previously been studied in a model of fatty liver, although they have been shown to limit hepatic fibrosis induced by bile duct ligation (Eken et al. 2006;Ozturk et al. 2006), a model known to influence hepatic glucocorticoid metabolism via bile acid induced inhibition of 5β-reductase activity, an enzyme also important in cholesterol and bile acid metabolism (McNeilly et al. 2010). Glucocorticoids have a number of potential anti-fibrotic actions, including effects on hepatic stellate cells to reduce transforming growth factor-β secretion (Bolkenius et al. 2004) and type I collagen expression (Cutroneo and Sterling, Jr. 2004). Other possible anti-fibrotic mechanisms include reduced angiotensin 1 receptor expression in hepatoma cells (Wintersgill et al. 1995) and reduced expression of tissue inhibitor of matrix metalloproteinases (Oishi et al. 2002).

It is feasible that the predicted relative increase in hepatic glucocorticoid levels in CCl_4 treated MCDD mice may have limited the rise in mRNA levels of TIMP2 seen in CS and CDD mice (see Section 3.3.6).

Human studies are more problematic than rodent studies of NAFLD due to the heterogeneity of subjects, the chronicity of changes leading to steatohepatitis and/or fibrosis, and the need for invasive liver biopsy. In a recent study, currently only published in abstract form (Ahmed et al. 2010), patients with biopsy proven NAFLD had increased 5α-reductase and reduced 11β-HSD1 activity, thus seeming to limit hepatic glucocorticoid exposure. Interestingly, hepatic 11β-HSD1 activity was significantly increased in the subgroup with steatohepatitis. This is in contrast to the gene expression results in our MCDD mice, although this could relate to the stage of NAFLD pathogenesis, with the increase in 11β-HSD1 activity protecting against progression to fibrosis. However, another recent study of 75 patients found no association with 11β-HSD1 activity and steatohepatitis, whilst 5α and 5β-reductase activity was increased in patients with NAFLD, both with and without NASH, versus controls undergoing investigation for increased aminotransferases (Konopelska et al. 2009). Other evidence for an important role of abnormalities of glucocorticoid metabolism in the pathogenesis of NAFLD comes from a study demonstrating increased hepatic glucocorticoid clearance and post dexamethasone suppression of cortisol levels correlating with the degree of hepatic inflammation and fibrosis in NAFLD, although metabolism of dexamethasone in NAFLD is a potential confounder (Targher et al. 2006).

Our study clearly has a number of limitations, including the disparity between the gene expression and enzyme activity results which, in other studies have generally correlated well e.g. (Livingstone et al. 2009a;Livingstone et al. 2000b). We have not examined glucocorticoid metabolism in adipose tissue, which may be an important determinant of hepatic glucocorticoid signalling, nor have we measured expression of the gene encoding the glucocorticoid receptor. In addition we have not measured circulating plasma corticosterone levels, which are likely to be increased following the CCl_4 insult (Swain et al. 1999).

In summary, we have undertaken preliminary studies to investigate glucocorticoid metabolism in two contrasting models of NAFLD, with and without steatohepatitis, and demonstrated changes in gene expression of a number of key enzymes regulating hepatic glucocorticoid levels, including in response to CCl_4 injury. In particular, suppression of hepatic 11β-HSD1 was an important finding which may contribute to the development of steatohepatitis in the MCDD model, whilst upregulation of 11β-HSD1 in response to a toxic fibrogenic insult may limit progression to hepatic fibrosis. Given the abnormalities of hepatic glucocorticoid metabolism in human obesity, and the potential therapeutic use of 11β-HSD1 in type 2 diabetes, these findings may have important implications for the development of progressive liver disease in humans. Indeed, I have proceeded to a clinical study investigating the role of glucocorticoid blockade and its influence on fatty acid metabolism in individuals with type 2 diabetes and fatty liver.

6 Chapter 6 - Conclusions

The increasing prevalence of obesity represents a major public health concern due to the associated morbidity and mortality. Not only does obesity lead to a multitude of chronic health problems including hypertension, dyslipidaemia, type 2 diabetes, obstructive sleep apnoea, and premature cardiovascular disease, but it is also associated with increased accumulation of fat within the liver or non-alcoholic fatty liver disease (NAFLD). In its simplest form, steatosis, NAFLD may be relatively benign and indeed may actually be a protective mechanism against the metabolic consequences of obesity. However, for reasons that are unclear, some individuals also develop hepatic inflammation known as non-alcoholic steatohepatitis (NASH), which can progress to liver fibrosis, and cirrhosis, with associated increases in morbidity and mortality. Indeed, the majority of patients previously labelled as having cryptogenic cirrhosis are likely to have NAFLD.

We sought to investigate pathways influencing the development of progressive liver disease in NAFLD, hypothesising that abnormalities of fatty acid and glucocorticoid metabolism, promoting increased intrahepatic fatty acids and reduced intrahepatic glucocorticoids respectively, promote the development of steatohepatitis. Human studies of NAFLD are difficult to undertake due to the heterogeneity of patients, the difficulty in predicting those who will develop NASH and/or fibrosis, and the need to perform (ideally serial) liver biopsies, which have an associated morbidity and mortality. We therefore opted to study dietary models of NAFLD in rodents in which the risk of insulin resistance, steatohepatitis and fibrosis differs, namely the methionine and choline deficient diet (MCDD), and the isolated choline deficient diet (CDD). The MCDD is a commonly employed model of steatohepatitis, which can lead to mild fibrosis, whereas the CDD leads to steatosis alone and does not produce spontaneous fibrosis in mice. Previous studies have also shown these models to have unique properties with respect to hepatic insulin signalling, with hepatic insulin resistance despite marked weight loss in MCDD mice (Leclercq et al.

2007;Schattenberg et al. 2005), and protection from high fat diet induced insulin resistance in CDD mice (Raubenheimer et al. 2006).

Table 6.1 summarises the findings of our studies which may underlie the contrasting susceptibility to steatohepatitis in the MCDD model. In Chapter 3, after firstly confirming that a MCDD alone induced significant steatohepatitis, we investigated the possible molecular mechanisms underlying the contrasting predisposition to NASH and hepatic insulin resistance in these two dietary models of NAFLD and investigated whether these differences also influenced susceptibility to hepatic fibrosis induced by carbon tetrachloride (CCl_4). There were clear differences between CDD and MCDD fed mice, with marked upregulation of proinflammatory (e.g. TNFα, IL-1β) and profibrotic cytokine gene (e.g. TGFβ) expression only in the steatohepatitic MCDD livers. Consistent with a profibrotic phenotype there was also increased activation of activated hepatic stellate cells in the MCDD mice alone, with no significant change seen in CDD mice. However, CCl_4 did not augment proinflammatory or profibrotic gene expression in either model of NAFLD, and despite the widespread changes in the MCDD livers, surprisingly there was no increased susceptibility to CCl_4 induced hepatic fibrosis. Indeed, picosirius red staining appeared less pronounced in MCDD versus CDD livers, although it would have been optimal to have confirmed this with additional measures of hepatic fibrosis such as measures of hepatic hydroxproline concentration or histological scoring. .

Dynamic changes in matrix metalloproteinase (MMP) and their tissue inhibitors (TIMPs) play a central role in determining the fibrotic content of extracellular matrix and there were significant differences in MMP and TIMP expression between CDD and MCDD mice. The marked upregulation of MMPs in the MCDD versus CDD mice may have influenced fibrogenesis in the MCDD model, with an increased balance of metalloproteinase activity favouring the resolution of fibosis.

Table 6.1 Summary of findings in choline ± methionine deficieny in C57Bl6 mice

	CDD	MCDD
Liver TGs	↑	↑
Liver inflammation	↔	↑
Body weight	↔	↓
Susceptibility to liver fibrosis	↔	↔
Fatty acid metabolism		
FFA turnover	↔	↔
De novo lipogenesis	↔	↑
Hepatic TG export	↔	↓
FFA oxidation	?	?
Hepatic glucocorticoid metabolism (effects of CCl_4 in parenthesis)*		
11β-HSD1	↔ (↔)	↓ (relative ↑)
5α-Reductase	↔ (↔)	↓ (↓)
5β-Reductase	↔ (↑)	↔ (↑)
3α-HSD	↔ (↔)	↔ (↔)

*based on gene expression data

Simplistically, NAFLD can be considered an imbalance between the supply of free fatty acids (FFA), from either plasma (via lipolysis or dietary sources) or hepatic *de novo* lipogenesis, and the disposal of FFAs through hepatic VLDL export or fatty acid oxidation. However, despite being an important fuel, fatty acids are inherently toxic to hepatocytes, and if the capacity for FFA disposal is overwhelmed then this may promote the development of NASH and/or fibrosis. Hence, we hypothesised that the contrasting liver disease phenotypes in MCDD and CDD mice associate with differences in intrahepatic fatty acid metabolism.

To investigate this hypothesis, in Chapter 4 we undertook detailed metabolic studies, including the use of *in vivo* metabolic tracer techniques, in CDD and MCDD fed mice, as well as controls fed a supplemented diet. We demonstrated key differences in fatty acid metabolism which associated with the differing susceptibility to

steatohepatitis in MCDD and CDD fed mice. In particular, hepatic TG export was reduced in MCDD but not CDD mice, supporting recent *ex vivo* studies that VLDL export is unchanged in isolated choline deficiency, and contradicting a commonly reported belief that VLDL export is similarly reduced in both CDD and MCDD mice. In addition, our studies using mass isotopmer distribution analysis demonstrated an increase in *de novo* lipogenesis in MCDD mice alone. Neither model had an increased uptake of circulating FFAs. These results support the hypothesis that increased intrahepatic fatty acids promote steatohepatitis, in the MCDD model due to the combination of increased hepatic *de novo* lipogenesis and impaired VLDL export.

The final hypothesis was to investigate whether altered glucocorticoid metabolism, promoting a reduction in intrahepatic glucocorticoid levels, associates with susceptibility to hepatic insulin resistance, inflammation and fibrosis in NAFLD. This may represent an important mechanism as glucocorticoids are likely to be important regulators of both the metabolic and inflammatory response in NAFLD. There is accruing evidence that tissue specific dysregulation of glucocorticoid metabolism contributes to the metabolic complications of obesity, including insulin resistance. *In vivo* the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) acts to amplify local glucocorticoid levels, predominantly in the liver and adipose tissue. However, its activity is dysregulated in human obesity, with down regulation of hepatic 11β-HSD1 activity, associated with increased clearance of glucocorticoids by inactivating A-ring reductases. These changes would be predicted to reduce intrahepatic glucocorticoid concentrations, which given the metabolic and immunomodulatory effects of glucocorticoids may be critical in the pathogenesis of NAFLD.

We found changes suggestive of significant differences in glucocorticoid metabolism in the contrasting dietary models of NAFLD, although these need to be interpreted with caution as gene expression and enzyme activity assay results were not concordant. In association with steatohepatitis, 11β-HSD1 gene expression was reduced in the MCDD fed mice, whereas its expression was unchanged in CDD mice. Other rodent models incorporating obesity, insulin resistance and fatty liver (albeit

without steatohepatitis) have also generally found reduced hepatic 11β-HSD1 expression, but glucocorticoid clearance by inactivating A-ring reductases is usually increased. This was not the case in MCDD mice in which 5α-reductase expression was also reduced, perhaps compensating for the reduction in intrahepatic glucocorticoid levels in the MCDD model. These contrasting findings make it difficult to accurately predict the true influence of the MCDD on hepatic glucocorticoid levels.

That hepatic inflammation is not a feature of other rodent models of fatty liver in which 11β-HSD1 expression is reduced is important as it suggests that the predicted reduction in intrahepatic glucocorticoid levels may predispose to steatohepatitis, requiring the appropriate metabolic milieu or second hit to occur for inflammation to develop. Our results suggest the accumulation of intrahepatic fatty acids in the MCDD model, due to increased *de novo* lipogenesis, combined with impaired TG export, appear to be an important factor in contributing to this metabolic milieu predisposing to steatohepatitis and may have important implications for human obesity in which hepatic 11β-HSD1 activity is also reduced. Furthermore, it may be of particular importance if inhibitors of 11β-HSD1 are to be introduced as a treatment for trpe 2 diabetes.

The lack of suppression of 11β-HSD1 in the CDD livers also appears to be unique amongst models of NAFLD, suggesting that peripheral glucocorticoid regeneration may not be increased in this weight neutral model of NAFLD. Preservation of intrahepatic glucocorticoid levels could protect CDD fed mice from the development of steatohepatitis, although any increase in glucocorticoid levels would be predicted to have adverse metabolic consequences and indeed may actually contribute to the development of fatty liver. In contrast, CDD mice have previously been found to be protected from the metabolic effects of a high fat diet (Raubenheimer et al. 2006), although a failure to suppress intrahepatic glucocorticoid levels would be predicted to do the opposite (Stimson et al. 2010).

In addition to influencing susceptibility to hepatic inflammation, changes in hepatic glucocorticoid metabolism in response to CCl_4 may have significantly influenced the progression to hepatic fibrosis in CDD and MCDD mice. The MCDD model was unique in that 11β-HSD1 expression was upregulated in response to CCl_4, possibly limiting the fibrogenic effect of CCl_4 in these mice. However, this antifibrotic response may have detrimental metabolic effects on hepatic insulin resistance as recent human studies suggest that hepatic down regulation of 11β-HSD1 is lost in patients with type 2 diabetes, a population known to be at increased risk of NASH (Stimson et al. 2010).

Overall, these findings suggest that the downregulation of hepatic 11β-HSD1 in association with fatty liver acts may act as a protective mechanism against the development of insulin resistance and glucocorticoid hormone excess. This may then increase susceptibility to hepatic inflammation when other metabolic factors come in to play, such as an overwhelming load of intrahepatic fatty acids. However, the ability to upregulate 11β-HSD1 in response to CCl_4 in MCDD mice suggests this may be an important anti-fibrotic mechanism, although it is unclear whether this has any detrimental metabolic effect. Furthermore, the results need to be interpreted with caution given the disparity between the gene expression and the enzyme activity assay results. However, it is tempting to hypothesise that the reduced intrahepatic glucocorticoid levels are maladaptive, contributing to the reduced hepatic VLDL export, with any associated upregulation of fatty acid oxidation (Berthiaume et al. 2007b) overwhelming already saturated mitochondrial β-oxidation in MCDD fed mice.

The results of these experiments in rodents emphasise that further work is required to dissect the underlying mechanisms influencing progression of NAFLD to both steatohepatitis and fibrosis. These results have provided interesting preliminary data to support future studies, but the models were perhaps too disparate to draw definitive conclusions on the role of fatty acid and glucocorticoid metabolism on influencing progressive liver disease. To further test the hypothesis that abnormalities of fatty acid metabolism contribute to the progression of NAFLD

studies could be undertaken in humans with NASH and/or fibrosis to quantify hepatic *de novo* lipogenesis. VLDL export could also be quantified using metabolic tracer studies in humans, employing labelled acetate, or deuterium, as well as leucine tracers to calculate the contribution of *de novo* lipogenesis (Diraison et al. 2003).

A number of additional studies could be undertaken to further investigate the role of glucocorticoid hormone metabolism in the development of NASH and hepatic fibrosis. In the first instance, profiling urinary glucocorticoid metabolites or undertaking additional enzyme activity assays in the CDD and MCDD models may provide further evidence for a role in abnormalities of glucocorticoid metabolism in the development of progressive liver disease. Other studies which may help to delineate the effects of glucocorticoids on the development of steatohepatitis and fibrosis would include employing the same diets and/or CCl_4 injury in hepatic over-expressors of 11β-HSD1 or adrenalectomised mice. Alternatively, 11β-HSD1 or tissue-specific glucocorticoid receptor knockout mice, or pharmacological 11β-HSD1 inhibitors could be employed. However, in all these models it could be difficult to dissect which effects are secondary to the immunomodulatory versus the metabolic effects of glucocorticoids, the latter of which may require further metabolic tracer studies to be undertaken. Lastly, a long term prospective study of urinary steroid profiling in patients with NAFLD examining their subsequent risk of heptic inflammation and fibrosis would be desirable to investigate the above hypothesis.

To conclude, we have shown in rodent models that abnormalities of hepatic fatty acid metabolism are associated with the development of non-alcoholic steatohepatitis. However, despite these inflammatory changes we did not show an increased susceptibility to hepatic fibrosis, suggesting that additional factors are necessary for inflammation to be translated into fibrosis. Lastly, our preliminary results suggest that hepatic glucocorticoid metabolism may be a key regulator of susceptibility to inflammation and the progression to liver fibrosis. These studies justify undertaking further research to determine the relevance of these findings in humans.

Bibliography

1. Aarsaether, N., R. K. Berge, A. Aarsland, A. M. Svardal, and P. M. Ueland. 1988. Effect of methotrexate on long-chain fatty acid metabolism in liver of rats fed a standard or a defined, choline-deficient diet. Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism 958: 70-80.

2. Adachi, M., E. Seki, T. Hibi, and D. A. Brenner. 2008. 364 High Molecular Weight Adiponectin Inhibits Proliferation of Hepatic Stellate Cells Via Activation of AMP-Activated Protein Kinase (Ampk). Gastroenterology 134: A-764.

3. Adams, L. A., P. Angulo, and K. D. Lindor. 2005. Nonalcoholic fatty liver disease. CMAJ. 172: 899-905.

4. Addison, T. 1855. On the constitutional and local effects of disease of the supra-renal capsules. London: reprinted by S Highley.

5. Agius, L., M. H. Chowdhury, and K. G. Alberti. 1986. Regulation of ketogenesis, gluconeogenesis and the mitochondrial redox state by dexamethasone in hepatocyte monolayer cultures. Biochem. J 239: 593-601.

6. Ahmed, A., Brady, T., Brown, C., Guest, P., Wagenmakers, A., Newsone, P., Hubscher, S., Elias, E., Adams, D., Tomlinson, J. W., and Stewart, P. M. Cortisol metabolism and hepatic expression of 11â-hydroxysteroid dehydrogenase type 1 in patients with non-alcoholic fatty liver disease. Medical Research Society meeting , 13P. 2010.
Ref Type: Abstract

7. Alberti, L., A. Girola, L. Gilardini, A. Conti, S. Cattaldo, G. Micheletto, and C. Invitti. 2007. Type 2 diabetes and metabolic syndrome are associated with increased expression of 11beta-hydroxysteroid dehydrogenase 1 in obese subjects. Int. J Obes. (Lond) 31: 1826-1831.

8. Ametaj, B. N., G. Bobe, Y. Lu, J. W. Young, and D. C. Beitz. 2003. Effect of sample preparation, length of time, and sample size on quantification of total lipids from bovine liver. J Agric. Food Chem. 51: 2105-2110.

9. Andrew, R., D. I. W. Phillips, and B. R. Walker. 1998. Obesity and gender influence cortisol secretion and metabolism in man. J. Clin. Endocrinol. Metab. 83: 1806-1809.

10. Andrews, R. C. and B. R. Walker. 1999. Glucocorticoids and insulin resistance: old hormones, new targets. Clin. Sci. 96: 513-523.

11. Angulo, P., J. C. Keach, K. P. Batts, and K. D. Lindor. 1999. Independent predictors of liver fibrosis in patients with nonalcoholic steatohepatitis. Hepatology 30: 1356-1362.

12. Anstee, Q. M. and R. D. Goldin. 2006. Mouse models in non-alcoholic fatty liver disease and steatohepatitis research. International Journal of Experimental Pathology 87: 1-16.

13. Appel, B. and S. K. Fried. 1992. Effects of insulin and dexamethasone on lipoprotein lipase in human adipose tissue. Am J Physiol 262: E695-E699.

14. Arsov, T., C. Z. Larter, C. J. Nolan, N. Petrovsky, C. C. Goodnow, N. C. Teoh, M. M. Yeh, and G. C. Farrell. 2006. Adaptive failure to high-fat diet characterizes steatohepatitis in Alms1 mutant mice. Biochem. Biophys. Res. Commun. 342: 1152-1159.

15. Arthur, M. J. Fibrogenesis II. Metalloproteinases and their inhibitors in liver fibrosis. AJP - Gastrointestinal and Liver Physiology 279[2], G245-G249. 2000.

16. Atzori, L., G. Poli, and A. Perra. 2009. Hepatic stellate cell: a star cell in the liver. Int. J Biochem. Cell Biol 41: 1639-1642.

17. Baar, R. A., C. S. Dingfelder, L. A. Smith, D. A. Bernlohr, C. Wu, A. J. Lange, and E. J. Parks. 2005. Investigation of in vivo fatty acid metabolism in AFABP/aP2(-/-) mice. Am. J. Physiol Endocrinol. Metab 288: E187-E193.

18. Barat, P., D. E. Livingstone, C. M. Elferink, C. R. McDonnell, B. R. Walker, and R. Andrew. 2007. Effects of gonadectomy on glucocorticoid metabolism in obese Zucker rats. Endocrinology 148: 4836-4843.

19. Barnes, P. J. 1998. Anti-inflammatory actions of glucocorticoids: molecular mechanisms. Clin Sci. (Lond) 94: 557-572.

20. Bartels, E. D., M. Lauritsen, and L. B. Nielsen. 2002. Hepatic expression of microsomal triglyceride transfer protein and in vivo secretion of triglyceride-rich lipoproteins are increased in obese diabetic mice. Diabetes 51: 1233-1239.

21. Basu, A., R. Basu, P. Shah, A. Vella, R. A. Rizza, and M. D. Jensen. 2001. Systemic and regional free fatty acid metabolism in type 2 diabetes. Am J Physiol Endocrinol. Metab 280: E1000-E1006.

22. Bataller, R. and D. A. Brenner. 2005. Liver fibrosis. J Clin Invest 115: 209-218.

23. Bates, E. J. and E. D. Saggerson. 1979. A study of the glycerol phosphate acyltransferase and dihydroxyacetone phosphate acyltransferase activities in rat liver mitochondrial and microsomal fractions. Relative distribution in parenchymal and non-parenchymal cells, effects of N-ethylmaleimide, palmitoyl-coenzyme A concentration, starvation, adrenalectomy and anti-insulin serum treatment. Biochem. J 182: 751-762.

24. Baxter, J. D. and P. H. Forsham. 1972. Tissue effects of glucocorticoids. Am J Med. 53: 573-589.

25. Bederman, I. R., A. E. Reszko, T. Kasumov, F. David, D. H. Wasserman, J. K. Kelleher, and H. Brunengraber. 2004. Zonation of labeling of lipogenic acetyl-CoA across the liver: implications for studies of lipogenesis by mass isotopomer analysis. J Biol Chem. 279: 43207-43216.

26. Benyon, R. C., J. P. Iredale, S. Goddard, P. J. Winwood, and M. J. Arthur. 1996. Expression of tissue inhibitor of metalloproteinases 1 and 2 is increased in fibrotic human liver. Gastroenterology 110: 821-831.

27. Berdanier, C. D. 1989. Role of glucocorticoids in the regulation of lipogenesis. FASEB J 3: 2179-2183.

28. Bergmeyer, H. U., P. Scheibe, and A. W. Wahlefeld. 1978. Optimization of methods for aspartate aminotransferase and alanine aminotransferase. Clin Chem. 24: 58-73.

29. Berthiaume, M., M. Laplante, W. Festuccia, Y. Gelinas, S. Poulin, J. Lalonde, D. R. Joanisse, R. Thieringer, and Y. Deshaies. 2007a. Depot-specific modulation of rat intraabdominal adipose tissue lipid metabolism by pharmacological inhibition of 11beta-hydroxysteroid dehydrogenase type 1. Endocrinology 148: 2391-2397.

30. Berthiaume, M., M. Laplante, W. T. L. Festuccia, K. Cianflone, L. P. Turcotte, D. R. Joanisse, G. Olivecrona, R. Thieringer, and Y. Deshaies. 2007b. 11{beta}-HSD1 inhibition improves triglyceridemia through reduced liver VLDL secretion and partitions lipids towards oxidative tissues. American Journal of Physiology - Endocrinology & Metabolism **293**: E1045-E1052.

31. Best, C. H., J. M. Hershey, and M. E. Huntsman. 1932. The effect of lecithine on fat deposition in the liver of the normal rat. J Physiol 75: 56-66.

32. Biddinger, S. B., M. Miyazaki, J. Boucher, J. M. Ntambi, and C. R. Kahn. 2006. Leptin suppresses stearoyl-CoA desaturase 1 by mechanisms independent of insulin and sterol regulatory element-binding protein-1c. Diabetes 55: 2032-2041.

33. Birkenhager, J. C., H. A. Timmermans, and S. W. Lamberts. 1976. Depressed plasma FFA turnover rate in Cushing's syndrome. J Clin. Endocrinol. Metab 42: 28-32.

34. Bjorkegren, J., A. Beigneux, M. O. Bergo, J. J. Maher, and S. G. Young. 2002. Blocking the secretion of hepatic very low density lipoproteins renders the liver more susceptible to toxin-induced injury. J Biol Chem. 277: 5476-5483.

35. Blaak, E. E., M. A. Van Baak, G. J. Kemerink, M. T. Pakbiers, G. A. Heidendal, and W. H. Saris. 1994. Beta-adrenergic stimulation of energy expenditure and forearm skeletal muscle metabolism in lean and obese men. Am J Physiol 267: E306-E315.

36. Boden, G. 1997. Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. Diabetes 46: 3-10.

37. Boden, G., X. Chen, E. Capulong, and M. Mozzoli. 2001. Effects of free fatty acids on gluconeogenesis and autoregulation of glucose production in type 2 diabetes. Diabetes 50: 810-816.

38. Boden, G., P. She, M. Mozzoli, P. Cheung, K. Gumireddy, P. Reddy, X. Xiang, Z. Luo, and N. Ruderman. 2005. Free fatty acids produce insulin resistance and activate the proinflammatory nuclear factor-kappaB pathway in rat liver. Diabetes 54: 3458-3465.

39. Boden, G. and G. I. Shulman. 2002. Free fatty acids in obesity and type 2 diabetes: defining their role in the development of insulin resistance and beta-cell dysfunction. Eur. J Clin. Invest 32 Suppl 3: 14-23.

40. Bolkenius, U., D. Hahn, A. M. Gressner, K. Breitkopf, S. Dooley, and L. Wickert. 2004. Glucocorticoids decrease the bioavailability of TGF-beta which leads to a reduced TGF-beta signaling in hepatic stellate cells. Biochem. Biophys. Res. Commun. 325: 1264-1270.

41. Boll, M., L. W. Weber, E. Becker, and A. Stampfl. 2001. Mechanism of carbon tetrachloride-induced hepatotoxicity. Hepatocellular damage by reactive carbon tetrachloride metabolites. Z. Naturforsch. C. 56: 649-659.

42. Borum, P. 2009. Handbook of vitamins. New York: Marcel Decker. 557-563 p.

43. Botto, M., C. Dell'Agnola, A. E. Bygrave, E. M. Thompson, H. T. Cook, F. Petry, M. Loos, P. P. Pandolfi, and M. J. Walport. 1998. Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. Nat. Genet. 19: 56-59.

44. Bradbury, M. W. and P. D. Berk. 2004. Lipid metabolism in hepatic steatosis. Clin Liver Dis. 8: 639-71, xi.

45. Brillon, D. J., B. Zheng, R. G. Campbell, and D. E. Matthews. 1995. Effect of cortisol on energy expenditure and amino acid metabolism in humans. Am J Physiol 268: E501-E513.

46. Browning, J. D., L. S. Szczepaniak, R. Dobbins, P. Nuremberg, J. D. Horton, J. C. Cohen, S. M. Grundy, and H. H. Hobbs. 2004. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. Hepatology 40: 1387-1395.

47. Brunt, E. M., C. G. Janney, A. M. Di Bisceglie, B. A. Neuschwander-Tetri, and B. R. Bacon. 1999. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. Am J Gastroenterol. 94: 2467-2474.

48. Bugianesi, E., U. Pagotto, R. Manini, E. Vanni, A. Gastaldelli, I. R. de, E. Gentilcore, S. Natale, M. Cassader, M. Rizzetto, R. Pasquali, and G.

Marchesini. 2005. Plasma adiponectin in nonalcoholic fatty liver is related to hepatic insulin resistance and hepatic fat content, not to liver disease severity. J Clin Endocrinol Metab 90: 3498-3504.

49. Burdge, G. C., P. Wright, A. E. Jones, and S. A. Wootton. 2000. A method for separation of phosphatidylcholine, triacylglycerol, non-esterified fatty acids and cholesterol esters from plasma by solid-phase extraction. Br. J Nutr. 84: 781-787.

50. Burt, M. G., J. Gibney, and K. K. Ho. 2006. Characterization of the metabolic phenotypes of Cushing's syndrome and growth hormone deficiency: a study of body composition and energy metabolism. Clin Endocrinol (Oxf) 64: 436-443.

51. Burt, M. G., J. Gibney, and K. K. Y. Ho. 2007. Protein metabolism in glucocorticoid excess: study in Cushing's syndrome and the effect of treatment. Am J Physiol Endocrinol Metab 292: E1426-E1432.

52. Campbell, P. J., M. G. Carlson, J. O. Hill, and N. Nurjhan. 1992. Regulation of free fatty acid metabolism by insulin in humans: role of lipolysis and reesterification. Am J Physiol 263: E1063-E1069.

53. Carmiel-Haggai, M., A. I. Cederbaum, and N. Nieto. 2005. A high-fat diet leads to the progression of non-alcoholic fatty liver disease in obese rats. FASEB J 19: 136-138.

54. Carpentier, A., C. Taghibiglou, N. Leung, L. Szeto, S. Van Iderstine, K. Uffelman, R. Buckingham, K. Adeli, and G. F. Lewis. 2002. Ameliorated hepatic insulin resistance is associated with normalization of microsomal triglyceride transfer protein expression and reduction in VLDL assembly and secretion in the fructose-fed hamster. J. Biol. Chem. M204568200.

55. Carter, A. L. and R. Frenkel. 1978. The relationship of choline and carnitine in the choline deficient rat. J Nutr. 108: 1748-1754.

56. Chapman, K. E., A. E. Coutinho, M. Gray, J. S. Gilmour, J. S. Savill, and J. R. Seckl. 2009. The role and regulation of 11beta-hydroxysteroid dehydrogenase type 1 in the inflammatory response. Mol. Cell Endocrinol 301: 123-131.

57. Charles, M. A., E. Eschwege, N. Thibult, J. R. Claude, J. M. Warnet, G. E. Rosselin, J. Girard, and B. Balkau. 1997. The role of non-esterified fatty acids in the deterioration of glucose tolerance in Caucasian subjects: results of the Paris Prospective Study. Diabetologia 40: 1101-1106.

58. Charlton, M., R. Sreekumar, D. Rasmussen, K. Lindor, and K. S. Nair. 2002. Apolipoprotein synthesis in nonalcoholic steatohepatitis. Hepatology 35: 898-904.

59. Chen, X., N. Iqbal, and G. Boden. 1999. The effects of free fatty acids on gluconeogenesis and glycogenolysis in normal subjects. J Clin. Invest 103: 365-372.

60. Chen, Y., Y. Yang, M. L. Miller, D. Shen, H. G. Shertzer, K. F. Stringer, B. Wang, S. N. Schneider, D. W. Nebert, and T. P. Dalton. 2007. Hepatocyte-specific Gclc deletion leads to rapid onset of steatosis with mitochondrial injury and liver failure. Hepatology 45: 1118-1128.

61. Chinkes, D. L., A. Aarsland, J. Rosenblatt, and R. R. Wolfe. 1996. Comparison of mass isotopomer dilution methods used to compute VLDL production in vivo. Am J Physiol 271: E373-E383.

62. Choi, C. S., D. B. Savage, A. Kulkarni, X. X. Yu, Z. X. Liu, K. Morino, S. Kim, A. Distefano, V. T. Samuel, S. Neschen, D. Zhang, A. Wang, X. M. Zhang, M. Kahn, G. W. Cline, S. K. Pandey, J. G. Geisler, S. Bhanot, B. P. Monia, and G. I. Shulman. 2007. Suppression of Diacylglycerol Acyltransferase-2 (DGAT2), but Not DGAT1, with Antisense Oligonucleotides Reverses Diet-induced Hepatic Steatosis and Insulin Resistance. J. Biol. Chem. 282: 22678-22688.

63. Choi, S. and A. M. Diehl. 2005. Role of inflammation in nonalcoholic steatohepatitis. Curr. Opin. Gastroenterol. 21: 702-707.

64. Chong, P. K., R. T. Jung, C. M. Scrimgeour, and M. J. Rennie. 1994. The effect of pharmacological dosages of glucocorticoids on free living total energy expenditure in man. Clin. Endocrinol (Oxf) 40: 577-581.

65. Chowdhry, S., M. H. Nazmy, P. J. Meakin, A. T. nkova-Kostova, S. V. Walsh, T. Tsujita, J. F. Dillon, M. L. Ashford, and J. D. Hayes. 2010. Loss of Nrf2 markedly exacerbates nonalcoholic steatohepatitis. Free Radic. Biol Med. 48: 357-371.

66. Clark, J. M., F. L. Brancati, and A. M. Diehl. 2003. The prevalence and etiology of elevated aminotransferase levels in the United States. Am J Gastroenterol. 98: 960-967.

67. Clawson, G. A. 1989. Mechanisms of carbon tetrachloride hepatotoxicity. Pathol. Immunopathol. Res. 8: 104-112.

68. Clerc, D., H. Wick, and U. Keller. 1986. Acute cortisol excess results in unimpaired insulin action on lipolysis and branched chain amino acids, but not on glucose kinetics and C-peptide concentrations in man. Metabolism 35: 404-410.

69. Coburn, C. T., F. F. Knapp, Jr., M. Febbraio, A. L. Beets, R. L. Silverstein, and N. A. Abumrad. 2000. Defective uptake and utilization of long chain fatty acids in muscle and adipose tissues of CD36 knockout mice. J Biol Chem. 275: 32523-32529.

70. Consolo, M., A. Amoroso, D. A. Spandidos, and M. C. Mazzarino. 2009. Matrix metalloproteinases and their inhibitors as markers of inflammation and fibrosis in chronic liver disease (Review). Int. J Mol. Med. 24: 143-152.

71. Constandinou, C., N. Henderson, and J. P. Iredale. 2005. Modeling liver fibrosis in rodents. Methods Mol. Med. 117: 237-250.

72. Coppack, S. W., R. D. Evans, R. M. Fisher, K. N. Frayn, G. F. Gibbons, S. M. Humphreys, M. L. Kirk, J. L. Potts, and T. D. Hockaday. 1992. Adipose tissue metabolism in obesity: lipase action in vivo before and after a mixed meal. Metabolism 41: 264-272.

73. Coppack, S. W., M. D. Jensen, and J. M. Miles. 1994. In vivo regulation of lipolysis in humans. J Lipid Res. 35: 177-193.

74. Coutinho, A. E. and K. E. Chapman. 2010. The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. Mol. Cell Endocrinol.

75. Cuchel, M., L. T. Bloedon, P. O. Szapary, D. M. Kolansky, M. L. Wolfe, A. Sarkis, J. S. Millar, K. Ikewaki, E. S. Siegelman, R. E. Gregg, and D. J. Rader. 2007. Inhibition of microsomal triglyceride transfer protein in familial hypercholesterolemia. N. Engl. J Med. 356: 148-156.

76. Cushing, H. 1912. The pituitary body and its disorders. Philadelphia and London: Lippincott. 217 p.

77. Cutroneo, K. R. and K. M. Sterling, Jr. 2004. How do glucocorticoids compare to oligo decoys as inhibitors of collagen synthesis and potential toxicity of these therapeutics? J Cell Biochem. 92: 6-15.

78. Da Silva Morais, A., V. Lebrun, J. barca-Quinones, S. Brichard, L. Hue, B. Guigas, B. Viollet, and I. A. Leclercq. 2009. Prevention of steatohepatitis by pioglitazone: Implication of adiponectin-dependent inhibition of SREBP-1c and inflammation. Journal of Hepatology 50: 489-500.

79. Dallman, M. F., S. E. la Fleur, N. C. Pecoraro, F. Gomez, H. Houshyar, and S. F. Akana. 2004. Minireview: glucocorticoids--food intake, abdominal obesity, and wealthy nations in 2004. Endocrinology 145: 2633-2638.

80. Danielsson, H. and J. Sjovall. 1975. Bile acid metabolism. Annu. Rev. Biochem. 44: 233-253.

81. Day, C. P. and O. F. James. 1998. Steatohepatitis: a tale of two "hits"? Gastroenterology 114: 842-845.

82. de Alwis, N. M. and C. P. Day. 2008. Non-alcoholic fatty liver disease: the mist gradually clears. J Hepatol. 48 Suppl 1: S104-S112.

83. Dela, P. A., I. Leclercq, J. Field, J. George, B. Jones, and G. Farrell. 2005. NF-kappaB activation, rather than TNF, mediates hepatic inflammation in a murine dietary model of steatohepatitis. Gastroenterology 129: 1663-1674.

84. Deng, Q. G., H. She, J. H. Cheng, S. W. French, D. R. Koop, S. Xiong, and H. Tsukamoto. 2005. Steatohepatitis induced by intragastric overfeeding in mice. Hepatology 42: 905-914.

85. Desbriere, R., V. Vuaroqueaux, V. Achard, S. Boullu-Ciocca, M. Labuhn, A. Dutour, and M. Grino. 2006. 11beta-hydroxysteroid dehydrogenase type 1 mRNA is increased in both visceral and subcutaneous adipose tissue of obese patients. Obesity. (Silver. Spring) 14: 794-798.

86. Dienstag, J. L., R. D. Goldin, E. J. Heathcote, H. W. Hann, M. Woessner, S. L. Stephenson, S. Gardner, D. F. Gray, and E. R. Schiff. 2003. Histological outcome during long-term lamivudine therapy. Gastroenterology 124: 105-117.

87. Dinneen, S., A. Alzaid, J. Miles, and R. Rizza. 1993. Metabolic effects of the nocturnal rise in cortisol on carbohydrate metabolism in normal humans. J. Clin. Invest. 92: 2283-2290.

88. Diraison, F., P. Moulin, and M. Beylot. 2003. Contribution of hepatic de novo lipogenesis and reesterification of plasma non esterified fatty acids to plasma triglyceride synthesis during non-alcoholic fatty liver disease. Diabetes Metab 29: 478-485.

89. Divertie, G. D., M. D. Jensen, and J. M. Miles. 1991. Stimulation of lipolysis in humans by physiological hypercortisolemia. Diabetes 40: 1228-1232.

90. Djouadi, F., C. J. Weinheimer, J. E. Saffitz, C. Pitchford, J. Bastin, F. J. Gonzalez, and D. P. Kelly. 1998. A gender-related defect in lipid metabolism and glucose homeostasis in peroxisome proliferator- activated receptor alpha-deficient mice. J Clin Invest 102: 1083-1091.

91. Djurhuus, C. B., C. H. Gravholt, S. Nielsen, A. Mengel, J. S. Christiansen, O. E. Schmitz, and N. Moller. 2002. Effects of cortisol on lipolysis and regional interstitial glycerol levels in humans. American Journal of Physiology - Endocrinology & Metabolism 283: E172-E177.

92. Djurhuus, C. B., C. H. Gravholt, S. Nielsen, S. B. Pedersen, N. Moller, and O. Schmitz. 2004. Additive effects of cortisol and growth hormone on regional and systemic lipolysis in humans. American Journal of Physiology - Endocrinology & Metabolism 286: E488-E494.

93. Dole M. The relative atomic weight of oxygen in water and in air. J Am Chemical Society 57, 2731. 1935.
Ref Type: Generic

94. Dolinsky, V. W., D. N. Douglas, R. Lehner, and D. E. Vance. 2004. Regulation of the enzymes of hepatic microsomal triacylglycerol lipolysis and re-esterification by the glucocorticoid dexamethasone. Biochem. J 378: 967-974.

95. Donnelly, K. L., C. I. Smith, S. J. Schwarzenberg, J. Jessurun, M. D. Boldt, and E. J. Parks. 2005. Sources of fatty acids stored in liver and secreted via

lipoproteins in patients with nonalcoholic fatty liver disease. J Clin. Invest 115: 1343-1351.

96. Drake, A. J., D. E. W. Livingstone, R. Andrew, J. R. Seckl, N. M. Morton, and B. R. Walker. 2005. Reduced adipose glucocorticoid reactivation and increased hepatic glucocorticoid clearance as an early adaptation to high fat feeding in rats. Endocrinology 146: 913-919.

97. Duffield, J. S., S. J. Forbes, C. M. Constandinou, S. Clay, M. Partolina, S. Vuthoori, S. Wu, R. Lang, and J. P. Iredale. 2005. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. J. Clin. Invest 115: 56-65.

98. Edens, N. K., R. L. Leibel, and J. Hirsch. 1990. Mechanism of free fatty acid re-esterification in human adipocytes in vitro. J Lipid Res. 31: 1423-1431.

99. Edmison, J. and A. J. McCullough. 2007. Pathogenesis of non-alcoholic steatohepatitis: human data. Clin Liver Dis. 11: 75-104, ix.

100. Eicheler, W., J. Seitz, M. Steinhoff, W. G. Forssmann, K. Adermann, and Aumuller. 1995. Distribution of rat hepatic steroid 5alpha-reductase 1 as shown by immunohistochemistry. Experimental & Clinical Endocrinology & Diabetes 103: 105-112.

101. Eken, H., H. Ozturk, H. Ozturk, and H. Buyukbayram. 2006. Dose-related effects of dexamethasone on liver damage due to bile duct ligation in rats. World J Gastroenterol. 12: 5379-5383.

102. Ekstedt, M., L. E. Franzen, U. L. Mathiesen, L. Thorelius, M. Holmqvist, G. Bodemar, and S. Kechagias. 2006. Long-term follow-up of patients with NAFLD and elevated liver enzymes. Hepatology 44: 865-873.

103. Elliott, P., R. F. Peters, and A. M. White. 1971. A study of the relationship between glucocorticoid-induced weight loss in rats and the activity of skeletal-muscle and cardiac-muscle ribosomes in vitro. Biochem. J 125: 106P-107P.

104. Eng, F. J. and S. L. Friedman. 2000. Fibrogenesis I. New insights into hepatic stellate cell activation: the simple becomes complex. Am J Physiol Gastrointest Liver Physiol 279: G7-11.

105. Escher, G., I. Galli, B. S. Vishwanath, B. M. Frey, and F. J. Frey. 1997. Tumor necrosis factor alpha and interleukin 1beta enhance the cortisone/cortisol shuttle. Journal of Experimental Medicine 186: 189-198.

106. Evans-Storms, R. B. and J. A. Cidlowski. 2000. Delineation of an antiapoptotic action of glucocorticoids in hepatoma cells: the role of nuclear factor-kappaB. Endocrinology 141: 1854-1862.

107. Fabbrini, E., D. deHaseth, S. Deivanayagam, B. S. Mohammed, B. E. Vitola, and S. Klein. 2009. Alterations in fatty acid kinetics in obese adolescents with increased intrahepatic triglyceride content. Obesity. (Silver. Spring) 17: 25-29.

108. Fabbrini, E., B. S. Mohammed, F. Magkos, K. M. Korenblat, B. W. Patterson, and S. Klein. 2008. Alterations in adipose tissue and hepatic lipid kinetics in obese men and women with nonalcoholic fatty liver disease. Gastroenterology 134: 424-431.

109. Fallowfield, J. A., M. Mizuno, T. J. Kendall, C. M. Constandinou, R. C. Benyon, J. S. Duffield, and J. P. Iredale. 2007. Scar-associated macrophages are a major source of hepatic matrix metalloproteinase-13 and facilitate the resolution of murine hepatic fibrosis. J Immunol. 178: 5288-5295.

110. Fan, C. Y., J. Pan, R. Chu, D. Lee, K. D. Kluckman, N. Usuda, I. Singh, A. V. Yeldandi, M. S. Rao, N. Maeda, and J. K. Reddy. 1996. Hepatocellular and Hepatic Peroxisomal Alterations in Mice with a Disrupted Peroxisomal Fatty Acyl-coenzyme A Oxidase Gene. J. Biol. Chem. 271: 24698-24710.

111. Feldstein, A. E., A. Canbay, M. E. Guicciardi, H. Higuchi, S. F. Bronk, and G. J. Gores. 2003. Diet associated hepatic steatosis sensitizes to Fas mediated liver injury in mice. J. Hepatol. 39: 978-983.

112. Feldstein, A. E., B. G. Papouchado, P. Angulo, S. Sanderson, L. Adams, and G. J. Gores. 2005. Hepatic stellate cells and fibrosis progression in patients with nonalcoholic fatty liver disease. Clin Gastroenterol. Hepatol. 3: 384-389.

113. Feldstein, A. E., N. W. Werneburg, A. Canbay, M. E. Guicciardi, S. F. Bronk, R. Rydzewski, L. J. Burgart, and G. J. Gores. 2004. Free fatty acids promote hepatic lipotoxicity by stimulating TNF-alpha expression via a lysosomal pathway. Hepatology 40: 185-194.

114. Fielding, B. A. and K. N. Frayn. 1998. Lipoprotein lipase and the disposition of dietary fatty acids. Br. J Nutr 80: 495-502.

115. Folch, J., M. Lees, and G. H. S. Stanley. 1957. A simple method for the isloation and purification of total lipides from animal tissues. J. Biol. Chem. 226: 497-509.

116. Forbes, S. J., F. P. Russo, V. Rey, P. Burra, M. Rugge, N. A. Wright, and M. R. Alison. 2004. A significant proportion of myofibroblasts are of bone marrow origin in human liver fibrosis. Gastroenterology 126: 955-963.

117. Forrest, E. H., A. J. Morris, S. Stewart, M. Phillips, Y. H. Oo, N. C. Fisher, G. Haydon, J. O'Grady, and C. P. Day. 2007. The Glasgow alcoholic hepatitis score identifies patients who may benefit from corticosteroids. Gut 56: 1743-1746.

118. Frayn, K. N. 1983. Calculation of substrate oxidation rates in vivo from gaseous exchange. J Appl. Physiol 55: 628-634.

119. Frayn, K. N. 2002. Adipose tissue as a buffer for daily lipid flux. Diabetologia 45: 1201-1210.

120. Frayn, K. N., B. A. Fielding, and L. K. Summers. 1997. Investigation of human adipose tissue metabolism in vivo. J Endocrinol 155: 187-189.

121. Fried, S. K., C. D. Russell, N. L. Grauso, and R. E. Brolin. 1993. Lipoprotein lipase regulation by insulin and glucocorticoid in subcutaneous and omental adipose tissues of obese women and men. J Clin Invest 92: 2191-2198.

122. Friedman, S. L. 2005. Mac the knife? Macrophages- the double-edged sword of hepatic fibrosis. J Clin Invest 115: 29-32.

123. Fromenty, B. and D. Pessayre. 1995. Inhibition of mitochondrial beta-oxidation as a mechanism of hepatotoxicity. Pharmacol. Ther. 67: 101-154.

124. Garfinkel, A. G., P. Nilsson-ehle, and M. C. Schotz. 1976. Regulation of lipoprotein lipase. Induction by insulin. Biochimica et Biophysica Acta. 424(2):264-73.

125. Gauthier, M. S., K. Couturier, J. G. Latour, and J. M. Lavoie. 2003. Concurrent exercise prevents high-fat-diet-induced macrovesicular hepatic steatosis. J. Appl. Physiol. 94: 2127-2134.

126. Geerts, A. 2001. History, heterogeneity, developmental biology, and functions of quiescent hepatic stellate cells. Semin. Liver Dis. 21: 311-335.

127. Gentile, C. L. and M. J. Pagliassotti. 2008. The role of fatty acids in the development and progression of nonalcoholic fatty liver disease. J Nutr Biochem. 19: 567-576.

128. Georgescu, E. F. 2008. Angiotensin receptor blockers in the treatment of NASH/NAFLD: could they be a first-class option? Adv. Ther. 25: 1141-1174.

129. Ghoshal, A. K. and E. Farber. 1993. Choline deficiency, lipotrope deficiency and the development of liver disease including liver cancer: a new perspective. Lab Invest 68: 255-260.

130. Giacchetti, G., E. Faloia, C. Sardu, M. A. Camilloni, B. Mariniello, C. Gatti, G. G. Garrapa, M. Guerrieri, and F. Mantero. 2000. Gene expression of angiotensinogen in adipose tissue of obese patients. Int. J Obes. Relat Metab Disord. 24 Suppl 2: S142-S143.

131. Gibbons, G. F., A. M. Brown, D. Wiggins, and R. Pease. 2002. The roles of insulin and fatty acids in the regulation of hepatic very-low-density lipoprotein assembly. J R. Soc. Med. 95 Suppl 42: 23-32.

132. Gibbons, G. F., D. Wiggins, A. M. Brown, and A. M. Hebbachi. 2004. Synthesis and function of hepatic very-low-density lipoprotein. Biochem. Soc. Trans. 32: 59-64.

133. Gil, B., M. A. Pajares, J. M. Mato, and L. Alvarez. 1997. Glucocorticoid regulation of hepatic S-adenosylmethionine synthetase gene expression. Endocrinology 138: 1251-1258.

134. Giles, K. M., K. Ross, A. G. Rossi, N. A. Hotchin, C. Haslett, and I. Dransfield. 2001. Glucocorticoid augmentation of macrophage capacity for phagocytosis of apoptotic cells is associated with reduced p130Cas expression, loss of paxillin/pyk2 phosphorylation, and high levels of active Rac. J Immunol. 167: 976-986.

135. Gilham, D., S. Ho, M. Rasouli, P. Martres, D. E. Vance, and R. Lehner. 2003. Inhibitors of hepatic microsomal triacylglycerol hydrolase decrease very low density lipoprotein secretion. FASEB J 17: 1685-1687.

136. Gilmour, J. S., A. E. Coutinho, J. F. Cailhier, T. Y. Man, M. Clay, G. Thomas, H. J. Harris, J. J. Mullins, J. R. Seckl, J. S. Savill, and K. E. Chapman. 2006. Local amplification of glucocorticoids by 11beta-hydroxysteroid dehydrogenase type 1 promotes macrophage phagocytosis of apoptotic leukocytes. J. Immunol. 176: 7605-7611.

137. Goedecke, J. H., D. J. Wake, N. S. Levitt, E. V. Lambert, M. R. Collins, N. M. Morton, R. Andrew, J. R. Seckl, and B. R. Walker. 2006. Glucocorticoid metabolism within superficial subcutaneous rather than visceral adipose tissue is associated with features of the metabolic syndrome in South African women. Clin Endocrinol (Oxf) 65: 81-87.

138. Goodman, S. I. 1980. An introduction to gas chromatography-mass spectrometry and the inherited organic acidemias. Am J Hum. Genet. 32: 781-792.

139. Goudriaan, J. R., V. E. Dahlmans, B. Teusink, D. M. Ouwens, M. Febbraio, J. A. Maassen, J. A. Romijn, L. M. Havekes, and P. J. Voshol. 2003. CD36 deficiency increases insulin sensitivity in muscle, but induces insulin resistance in the liver in mice. J Lipid Res. 44: 2270-2277.

140. Gravholt, C. H., R. Dall, J. S. Christiansen, N. Moller, and O. Schmitz. 2002. Preferential stimulation of abdominal subcutaneous lipolysis after prednisolone exposure in humans. Obes. Res. 10: 774-781.

141. Gudbrandsen, O. A., T. H. Rost, and R. K. Berge. 2006. Causes and prevention of tamoxifen-induced accumulation of triacylglycerol in rat liver. J Lipid Res. 47: 2223-2232.

142. Hadoke, P. W., J. Iqbal, and B. R. Walker. 2009. Therapeutic manipulation of glucocorticoid metabolism in cardiovascular disease. Br. J Pharmacol. 156: 689-712.

143. Hafezi-Moghadam, A., T. Simoncini, Z. Yang, F. P. Limbourg, J. C. Plumier, M. C. Rebsamen, C. M. Hsieh, D. S. Chui, K. L. Thomas, A. J. Prorock, V. E.

Laubach, M. A. Moskowitz, B. A. French, K. Ley, and J. K. Liao. 2002. Acute cardiovascular protective effects of corticosteroids are mediated by non-transcriptional activation of endothelial nitric oxide synthase. Nat. Med. 8: 473-479.

144. Hammond, L. E., P. A. Gallagher, S. Wang, S. Hiller, K. D. Kluckman, E. L. Posey-Marcos, N. Maeda, and R. A. Coleman. 2002. Mitochondrial Glycerol-3-Phosphate Acyltransferase-Deficient Mice Have Reduced Weight and Liver Triacylglycerol Content and Altered Glycerolipid Fatty Acid Composition. Mol. Cell. Biol. 22: 8204-8214.

145. Hammond, L. E., S. Neschen, A. J. Romanelli, G. W. Cline, O. R. Ilkayeva, G. I. Shulman, D. M. Muoio, and R. A. Coleman. 2005. Mitochondrial Glycerol-3-phosphate Acyltransferase-1 Is Essential in Liver for the Metabolism of Excess Acyl-CoAs. J. Biol. Chem. 280: 25629-25636.

146. Han, Y. P. 2006. Matrix metalloproteinases, the pros and cons, in liver fibrosis. J Gastroenterol. Hepatol. 21 Suppl 3: S88-S91.

147. Handoko, K., K. Yang, B. Strutt, W. Khalil, and D. Killinger. 2000. Insulin attenuates the stimulatory effects of tumor necrosis factor alpha on 11beta-hydroxysteroid dehydrogenase 1 in human adipose stromal cells. Journal of Steroid Biochemistry & Molecular Biology 72: 163-168.

148. Handy, J. A., N. K. Saxena, P. Fu, S. Lin, J. E. Mells, N. A. Gupta, and F. A. Anania. 2010. Adiponectin activation of AMPK disrupts leptin-mediated hepatic fibrosis via suppressors of cytokine signaling (SOCS-3). J Cell Biochem. 110: 1195-1207.

149. Hardy, R., E. H. Rabbitt, A. Filer, P. Emery, M. Hewison, P. M. Stewart, N. J. Gittoes, C. D. Buckley, K. Raza, and M. S. Cooper. 2008. Local and systemic glucocorticoid metabolism in inflammatory arthritis. Ann. Rheum. Dis. 67: 1204-1210.

150. Hellerstein, M. K. 1999. De novo lipogenesis in humans: metabolic and regulatory aspects. Eur. J Clin Nutr. 53 Suppl 1: S53-S65.

151. Hellerstein, M. K. and R. A. Neese. 1999. Mass isotopomer distribution analysis at eight years: theoretical, analytic, and experimental considerations. Am. J. Physiol 276: E1146-E1170.

152. Hellerstein, M. K. and R. A. Neese. 1992. Mass isotopomer distribution analysis: a technique for measuring biosynthesis and turnover of polymers. Am J Physiol 263: E988-1001.

153. Hemmann, S., J. Graf, M. Roderfeld, and E. Roeb. 2007. Expression of MMPs and TIMPs in liver fibrosis - a systematic review with special emphasis on anti-fibrotic strategies. J Hepatol. 46: 955-975.

154. Henderson, N. C. and J. P. Iredale. 2007. Liver fibrosis: cellular mechanisms of progression and resolution. Clin Sci. (Lond) 112: 265-280.

155. Hermanowski-Vosatka, A., J. M. Balkovec, K. Cheng, H. Y. Chen, M. Hernandez, G. C. Koo, C. B. Le Grand, Z. Li, J. M. Metzger, S. S. Mundt, H. Noonan, C. N. Nunes, S. H. Olson, B. Pikounis, N. Ren, N. Robertson, J. M. Schaeffer, K. Shah, M. S. Springer, A. M. Strack, M. Stowski, K. Wu, T. Wu, J. Xiao, B. B. Zhang, S. D. Wright, and R. Thieringer. 2005. 11β-HSD1 inhibition ameliorates metabolic syndrome and prevents progression of atherosclerosis in mice. Journal of Experimental Medicine 202: 517-527.

156. Hillgartner, F. B., L. M. Salati, and A. G. Goodridge. 1995. Physiological and molecular mechanisms involved in nutritional regulation of fatty acid synthesis. Physiol Rev. 75: 47-76.

157. Hoggard, N., J. G. Mercer, D. V. Rayner, K. Moar, P. Trayhurn, and L. M. Williams. 1997. Localization of leptin receptor mRNA splice variants in murine peripheral tissues by RT-PCR and in situ hybridization. Biochem. Biophys. Res. Commun. 232: 383-387.

158. Horie, Y., A. Suzuki, E. Kataoka, T. Sasaki, K. Hamada, J. Sasaki, K. Mizuno, G. Hasegawa, H. Kishimoto, M. Iizuka, M. Naito, K. Enomoto, S. Watanabe, T. W. Mak, and T. Nakano. 2004. Hepatocyte-specific Pten deficiency results in steatohepatitis and hepatocellular carcinomas. J Clin Invest 113: 1774-1783.

159. Huang, W., N. Dedousis, A. Bandi, G. D. Lopaschuk, and R. M. O'Doherty. 2006. Liver Triglyceride Secretion and Lipid Oxidative Metabolism Are Rapidly Altered by Leptin in Vivo. Endocrinology 147: 1480-1487.

160. Huang, W., N. Dedousis, and R. M. O'Doherty. 2007. Hepatic steatosis and plasma dyslipidemia induced by a high-sucrose diet are corrected by an acute leptin infusion. J. Appl. Physiol. 102: 2260-2265.

161. Hui, A. Y., V. W. Wong, H. L. Chan, C. T. Liew, J. L. Chan, F. K. Chan, and J. J. Sung. 2005. Histological progression of non-alcoholic fatty liver disease in Chinese patients. Aliment. Pharmacol. Ther. 21: 407-413.

162. Hui, J. M., A. Hodge, G. C. Farrell, J. G. Kench, A. Kriketos, and J. George. 2004. Beyond insulin resistance in NASH: TNF-alpha or adiponectin? Hepatology 40: 46-54.

163. Ibdah, J. A., P. Perlegas, Y. Zhao, J. Angdisen, H. Borgerink, M. K. Shadoan, J. D. Wagner, D. Matern, P. Rinaldo, and J. M. Cline. 2005. Mice heterozygous for a defect in mitochondrial trifunctional protein develop hepatic steatosis and insulin resistance. Gastroenterology 128: 1381-1390.

164. Iida, N., A. Sugiyama, H. Myoubudani, K. Inoue, M. Sugamata, T. Ihara, Y. Ueno, and F. Tashiro. 1998. Suppression of arachidonic acid cascade-mediated

apoptosis in aflatoxin B1-induced rat hepatoma cells by glucocorticoids. Carcinogenesis 19: 1191-1202.

165. Ikejima, K., K. Okumura, K. Kon, Y. Takei, and N. Sato. 2007. Role of adipocytokines in hepatic fibrogenesis. J Gastroenterol. Hepatol. 22 Suppl 1: S87-S92.

166. Ingalls, A. M., M. M. Dickie, and G. D. Snell. 1996. Obese, a new mutation in the house mouse. Obes. Res. 4: 101.

167. Ip, E., G. C. Farrell, G. Robertson, P. Hall, R. Kirsch, and I. Leclercq. 2003. Central role of PPARalpha-dependent hepatic lipid turnover in dietary steatohepatitis in mice. Hepatology 38: 123-132.

168. Iredale, J. P. 2007. Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ. J Clin Invest 117: 539-548.

169. Iredale, J. P., R. C. Benyon, J. Pickering, M. McCullen, M. Northrop, S. Pawley, C. Hovell, and M. J. Arthur. 1998. Mechanisms of spontaneous resolution of rat liver fibrosis. Hepatic stellate cell apoptosis and reduced hepatic expression of metalloproteinase inhibitors. J. Clin. Invest 102: 538-549.

170. Ishii, T., H. Masuzaki, T. Tanaka, N. Arai, S. Yasue, N. Kobayashi, T. Tomita, M. Noguchi, J. Fujikura, K. Ebihara, K. Hosoda, and K. Nakao. 2007. Augmentation of 11beta-hydroxysteroid dehydrogenase type 1 in LPS-activated J774.1 macrophages--role of 11beta-HSD1 in pro-inflammatory properties in macrophages. FEBS Lett. 581: 349-354.

171. Issa, R., X. Zhou, N. Trim, H. Millward-Sadler, S. Krane, C. Benyon, and J. Iredale. 2003. Mutation in collagen-1 that confers resistance to the action of collagenase results in failure of recovery from CCl4-induced liver fibrosis, persistence of activated hepatic stellate cells, and diminished hepatocyte regeneration. FASEB J 17: 47-49.

172. Itani, S. I., N. B. Ruderman, F. Schmieder, and G. Boden. 2002. Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and IkappaB-alpha. Diabetes 51: 2005-2011.

173. Ito, M., J. Suzuki, S. Tsujioka, M. Sasaki, A. Gomori, T. Shirakura, H. Hirose, M. Ito, A. Ishihara, H. Iwaasa, and A. Kanatani. 2007. Longitudinal analysis of murine steatohepatitis model induced by chronic exposure to high-fat diet. Hepatol. Res. 37: 50-57.

174. Jensen, M. D. 2003. Cytokine regulation of lipolysis in humans? J Clin Endocrinol Metab 88: 3003-3004.

175. Johnston, D. G., A. Gill, H. Orskov, G. F. Batstone, and K. G. Alberti. 1982. Metabolic effects of cortisol in man--studies with somatostatin. Metabolism 31: 312-317.

176. Jou, J., S. S. Choi, and A. M. Diehl. 2008. Mechanisms of disease progression in nonalcoholic fatty liver disease. Semin. Liver Dis. 28: 370-379.

177. Jung, H. R., S. M. Turner, R. A. Neese, S. G. Young, and M. K. Hellerstein. 1999. Metabolic adaptations to dietary fat malabsorption in chylomicron-deficient mice. Biochem. J 343 Pt 2: 473-478.

178. Kalant, D. and K. Cianflone. 2004. Regulation of fatty acid transport. Curr. Opin. Lipidol. 15: 309-314.

179. Kamada, Y., S. Tamura, S. Kiso, H. Matsumoto, Y. Saji, Y. Yoshida, K. Fukui, N. Maeda, H. Nishizawa, H. Nagaretani, Y. Okamoto, S. Kihara, J. Miyagawa, Y. Shinomura, T. Funahashi, and Y. Matsuzawa. 2003. Enhanced carbon tetrachloride-induced liver fibrosis in mice lacking adiponectin. Gastroenterology 125: 1796-1807.

180. Kannisto, K., K. H. Pietilainen, E. Ehrenborg, A. Rissanen, J. Kaprio, A. Hamsten, and H. Yki-Jarvinen. 2004. Overexpression of 11beta-hydroxy steroid dehydrogenase-1 in adipose tissue is associated with acquired obesity and features of insulin resistance: Studies in young adult monozygotic twins. J. Clin. Endocrinol. Metab. 89: 4414-4421.

181. Kapcala, L. P., T. Chautard, and R. L. Eskay. 1995. The protective role of the hypothalamic-pituitary-adrenal axis against lethality produced by immune, infectious, and inflammatory stress. Ann. N. Y. Acad. Sci. 771: 419-437.

182. Kashireddy, P. V. and M. S. Rao. 2004. Lack of peroxisome proliferator-activated receptor alpha in mice enhances methionine and choline deficient diet-induced steatohepatitis. Hepatol. Res. 30: 104-110.

183. Kelleher, J. K. and T. M. Masterson. 1992. Model equations for condensation biosynthesis using stable isotopes and radioisotopes. Am J Physiol 262: E118-E125.

184. Kelley, D. E., B. H. Goodpaster, and L. Storlien. 2002. Muscle triglyceride and insulin resistance. Annu. Rev. Nutr 22: 325-346.

185. Kersten, S., J. Seydoux, J. M. Peters, F. J. Gonzalez, B. Desvergne, and W. Wahli. 1999. Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. J Clin Invest 103: 1489-1498.

186. Kirsch, R., V. Clarkson, R. C. Verdonk, A. D. Marais, E. G. Shephard, B. Ryffel, and M. H. de la. 2006. Rodent nutritional model of steatohepatitis: effects of endotoxin (lipopolysaccharide) and tumor necrosis factor alpha deficiency. J. Gastroenterol. Hepatol. 21: 174-182.

187. Kleiner, D. E., E. M. Brunt, N. M. Van, C. Behling, M. J. Contos, O. W. Cummings, L. D. Ferrell, Y. C. Liu, M. S. Torbenson, A. Unalp-Arida, M. Yeh, A. J. McCullough, and A. J. Sanyal. 2005. Design and validation of a

histological scoring system for nonalcoholic fatty liver disease. Hepatology 41: 1313-1321.

188. Knapp, A. C., L. Todesco, M. Torok, K. Beier, and S. Krahenbuhl. 2008. Effect of carnitine deprivation on carnitine homeostasis and energy metabolism in mice with systemic carnitine deficiency. Ann. Nutr. Metab 52: 136-144.

189. Konopelska, S., T. Kienitz, B. Hughes, M. Pirlich, J. Bauditz, H. Lochs, C. J. Strasburger, P. M. Stewart, and M. Quinkler. 2009. Hepatic 11beta-HSD1 mRNA expression in fatty liver and nonalcoholic steatohepatitis. Clin Endocrinol (Oxf) 70: 554-560.

190. Koppe, S. W., A. Sahai, P. Malladi, P. F. Whitington, and R. M. Green. 2004. Pentoxifylline attenuates steatohepatitis induced by the methionine choline deficient diet. J Hepatol. 41: 592-598.

191. Koteish, A. and A. M. Diehl. 2001. Animal models of steatosis. Seminars in Liver Disease 21: 89-104.

192. Koteish, A. and A. M. Diehl. 2002. Animal models of steatohepatitis. Best Practice and Research Clinical Gastroenterology 16: 679-690.

193. Kotelevtsev, Y. V., M. C. Holmes, A. Burchell, P. M. Houston, D. Scholl, P. M. Jamieson, R. Best, R. W. Brown, C. R. W. Edwards, J. R. Seckl, and J. J. Mullins. 1997. 11β-Hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid inducible responses and resist hyperglycaemia on obesity and stress. Proc. Natnl. Acad. Sci. USA 94: 14924-14929.

194. Koutsari, C. and M. D. Jensen. 2006. Thematic review series: patient-oriented research. Free fatty acid metabolism in human obesity. J Lipid Res. 47: 1643-1650.

195. Kratky, D., R. Zimmermann, E. M. Wagner, J. G. Strauss, W. Jin, G. M. Kostner, G. Haemmerle, D. J. Rader, and R. Zechner. 2005. Endothelial lipase provides an alternative pathway for FFA uptake in lipoprotein lipase-deficient mouse adipose tissue. J Clin. Invest 115: 161-167.

196. Krebs, M. and M. Roden. 2005. Molecular mechanisms of lipid-induced insulin resistance in muscle, liver and vasculature. Diabetes Obes. Metab 7: 621-632.

197. Krsek, M., M. Rosicka, J. Nedvidkova, H. Kvasnickova, V. Hana, J. Marek, M. Haluzik, E. W. Lai, and K. Pacak. 2006. Increased lipolysis of subcutaneous abdominal adipose tissue and altered noradrenergic activity in patients with Cushing's syndrome: an in-vivo microdialysis study. Physiol Res. 55: 421-428.

198. Kulinski, A., D. E. Vance, and J. E. Vance. 2004. A choline-deficient diet in mice inhibits neither the CDP-choine pathway for phosphatidylcholine synthesis in hepatocytes nor apolipoprotein B secretion. J. Biol. Chem. 279: 23916-23924.

199. la Fleur, S. E., S. F. Akana, S. L. Manalo, and M. F. Dallman. 2004. Interaction between corticosterone and insulin in obesity: regulation of lard intake and fat stores. Endocrinology 145: 2174-2185.

200. Lacasa, D., B. Agli, and Y. Giudicelli. 1988. Permissive action of glucocorticoids on catecholamine-induced lipolysis: direct "in vitro" effects on the fat cell beta-adrenoreceptor-coupled-adenylate cyclase system. Biochem. Biophys. Res. Commun. 153: 489-497.

201. Lafontan, M. and N. Viguerie. 2006. Role of adipokines in the control of energy metabolism: focus on adiponectin. Curr. Opin. Pharmacol. 6: 580-585.

202. Lambert, K., G. Py, E. Robert, and J. Mercier. 2003. Does high-sucrose diet alter skeletal muscle and liver mitochondrial respiration? Horm. Metab Res. 35: 546-550.

203. Larter, C. Z. and G. C. Farrell. 2006. Insulin resistance, adiponectin, cytokines in NASH: Which is the best target to treat? J Hepatol. 44: 253-261.

204. Larter, C. Z., M. M. Yeh, W. G. Haigh, J. Williams, S. Brown, K. S. Bell-Anderson, S. P. Lee, and G. C. Farrell. 2008a. Hepatic free fatty acids accumulate in experimental steatohepatitis: role of adaptive pathways. J Hepatol. 48: 638-647.

205. Larter, C. Z., M. M. Yeh, J. Williams, K. S. Bell-Anderson, and G. C. Farrell. 2008b. MCD-induced steatohepatitis is associated with hepatic adiponectin resistance and adipogenic transformation of hepatocytes. J Hepatol. 49: 407-416.

206. Lau, D. C. and D. A. Roncari. 1983. Effects of glucocorticoid hormones on lipid-synthetic enzymes from different adipose tissue regions and from liver. Can. J Biochem. Cell Biol 61: 1245-1250.

207. Leclercq, I. A., G. C. Farrell, J. Field, D. R. Bell, F. J. Gonzalez, and G. R. Robertson. 2000. CYP2E1 and CYP4A as microsomal catalysts of lipid peroxides in murine nonalcoholic steatohepatitis. J Clin Invest 105: 1067-1075.

208. Leclercq, I. A., G. C. Farrell, R. Schriemer, and G. R. Robertson. 2002. Leptin is essental for the hepatic fibrogenic response to chronic liver injury. Journal of Hepatology 37: 206-213.

209. Leclercq, I. A., G. C. Farrell, C. Sempoux, A. Dela Pena, and Y. Horsmans. 2004. Curcumin inhibits NF-kappaB activation and reduces the severity of experimental steatohepatitis in mice. J Hepatol. 41: 926-934.

210. Leclercq, I. A., V. A. Lebrun, P. Starkel, and Y. J. Horsmans. 2007. Intrahepatic insulin resistance in a murine model of steatohepatitis: effect of PPARgamma agonist pioglitazone. Lab Invest 87: 56-65.

211. Lee, G. H., R. Proenca, J. M. Montez, K. M. Carroll, J. G. Darvishzadeh, J. I. Lee, and J. M. Friedman. 1996. Abnormal splicing of the leptin receptor in diabetic mice. Nature 379: 632-635.

212. Lee, W. N., E. A. Bergner, and Z. K. Guo. 1992. Mass isotopomer pattern and precursor-product relationship. Biol Mass Spectrom. 21: 114-122.

213. Letteron, P., N. Brahimi-Bourouina, M. A. Robin, A. Moreau, G. Feldman, and D. Passayre. 1997. Glucocorticoids inhibit mitochondrial matrix acyl-CoA dehydrogenases and fatty acid beta-oxidation. Am. J. Physiol. 272: G1141-G1150.

214. Letteron, P., A. Sutton, A. Mansouri, B. Fromenty, and D. Pessayre. 2003. Inhibition of microsomal triglyceride transfer protein: another mechanism for drug-induced steatosis in mice. Hepatology 38: 133-140.

215. Lewis, G. F., K. D. Uffelman, L. W. Szeto, B. Weller, and G. Steiner. 1995. Interaction between free fatty acids and insulin in the acute control of very low density lipoprotein production in humans. J Clin Invest 95: 158-166.

216. Li, X., S. M. Grundy, and S. B. Patel. 1997. Obesity in db and ob animals leads to impaired hepatic very low density lipoprotein secretion and differential secretion of apolipoprotein B-48 and B-100. J Lipid Res. 38: 1277-1288.

217. Li, Z. and D. E. Vance. 2008. Phosphatidylcholine and choline homeostasis. J Lipid Res. 49: 1187-1194.

218. Lieber, C. S., M. A. Leo, K. M. Mak, Y. Xu, Q. Cao, C. Ren, A. Ponomarenko, and L. M. DeCarli. 2004. Model of nonalcoholic steatohepatitis. Am J Clin Nutr 79: 502-509.

219. Lillioja, S., J. Foley, C. Bogardus, D. Mott, and B. V. Howard. 1986. Free fatty acid metabolism and obesity in man: in vivo in vitro comparisons. Metabolism 35: 505-514.

220. Lin, X., G. Schonfeld, P. Yue, and Z. Chen. 2002. Hepatic fatty acid synthesis is suppressed in mice with fatty livers due to targeted apolipoprotein B38.9 mutation. Arterioscler Thromb Vasc Biol 22: 476-482.

221. Linden, D., L. William-Olsson, M. Rhedin, A. K. Asztely, J. C. Clapham, and S. Schreyer. 2004. Overexpression of mitochondrial GPAT in rat hepatocytes leads to decreased fatty acid oxidation and increased glycerolipid biosynthesis. J. Lipid Res. 45: 1279-1288.

222. Linder, C., S. S. Chernick, T. R. Fleck, and R. O. Scow. 1976. Lipoprotein lipase and uptake of chylomicron triglyceride by skeletal muscle of rats. American Journal of Physiology. 231(3):860-4.

223. Lindsay, R. S., A. Tataranni, P. Permana, D. E. W. Livingstone, D. J. Wake, and B. R. Walker. 2003. Subcutaneous adipose 11β-hydroxysteroid

dehydrogenase type 1 activity and mRNA levels are associated with adiposity and insulinaemia in Pima Indians and Caucasians. J. Clin. Endocrinol. Metab. 88: 2738-2744.

224. Lionakis, M. S. and D. P. Kontoyiannis. 2003. Glucocorticoids and invasive fungal infections. Lancet 362: 1828-1838.

225. Liu, Y., Y. Nakagawa, Y. Wang, R. Li, X. Li, T. Ohzeki, and T. C. Friedman. 2003. Leptin activation of corticosterone production in hepatocytes may contribute to the reversal of obesity and hyperglycemia in leptin-deficient ob/ob mice. Diabetes 52: 1409-1416.

226. Liu, Y., Y. Nakagawa, Y. Wang, R. Sakurai, P. V. Tripathi, K. Lutfy, and T. C. Friedman. 2005. Increased glucocorticoid receptor and 11{beta}-hydroxysteroid dehydrogenase type 1 expression in hepatocytes may contribute to the phenotype of type 2 diabetes in db/db mice. Diabetes 54: 32-40.

227. Livingstone, D. E., S. L. Grassick, G. L. Currie, B. R. Walker, and R. Andrew. 2009a. Dysregulation of glucocorticoid metabolism in murine obesity: comparable effects of leptin resistance and deficiency. J Endocrinol 201: 211-218.

228. Livingstone, D. E., K. J. McInnes, B. R. Walker, and R. Andrew. 2005. Increased A-ring reduction of glucocorticoids in obese Zucker rats: effects of insulin sensitization. Obesity Research 13: 1523-1526.

229. Livingstone, D. E., Walker, B. R., and Andrew, R. Susceptibility to hyperinsulinaemia and fatty liver with loss of 5alpha-reductase 1 occurs in rats and mice and is not androgen dependent. Endocrine Abstracts [19], 136. 2009b. Ref Type: Abstract

230. Livingstone, D. E. W., G. C. Jones, K. Smith, R. Andrew, C. J. Kenyon, and B. R. Walker. 2000a. Understanding the role of glucocorticoids in obesity: tissue-specific alterations of corticosterone metabolism in obese Zucker rats. Endocrinology 141: 560-563.

231. Livingstone, D. E. W., C. J. Kenyon, and B. R. Walker. 2000b. Mechanisms of dysregulation of 11β-hydroxysteroid dehydrogenase type 1 in Zucker obese rats. J. Endocrinol. 167: 533-539.

232. Ljumovic, D., I. Diamantis, A. K. Alegakis, and E. A. Kouroumalis. 2004. Differential expression of matrix metalloproteinases in viral and non-viral chronic liver diseases. Clin Chim. Acta 349: 203-211.

233. Lombardi, B., P. Pani, and F. F. Schlunk. 1968. Choline-deficiency fatty liver: impaired release of hepatic triglycerides. J Lipid Res. 9: 437-446.

234. Lu, S. C. 2008. Regulation of glutathione synthesis. Mol. Aspects Med.

235. Lu, S. C., L. Alvarez, Z. Z. Huang, L. Chen, W. An, F. J. Corrales, M. a. A. Avila, G. Kanel, and J. M. Mato. 2001. Methionine adenosyltransferase 1A knockout mice are predisposed to liver injury and exhibit increased expression of genes involved in proliferation. Proceedings of the National Academy of Sciences of the United States of America 98: 5560-5565.

236. Ludwig, J., T. R. Viggiano, D. B. McGill, and B. J. Oh. 1980. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. Mayo Clin. Proc. 55: 434-438.

237. Luyckx, F. H., C. Desaive, A. Thiry, W. Dewe, A. J. Scheen, J. E. Gielen, and P. J. Lefebvre. 1998. Liver abnormalities in severely obese subjects: effect of drastic weight loss after gastroplasty. Int. J Obes. Relat Metab Disord. 22: 222-226.

238. Mai, K., T. Bobbert, V. Kullmann, J. Andres, V. Bahr, C. Maser-Gluth, H. Rochlitz, J. Spranger, S. Diederich, and A. F. Pfeiffer. 2006. No effect of free fatty acids on adrenocorticotropin and cortisol secretion in healthy young men. Metabolism 55: 1022-1028.

239. Mai, K., V. Kullmann, T. Bobbert, C. Maser-Gluth, M. Mohlig, V. Bahr, A. F. H. Pfeiffer, J. Spranger, and S. Diederich. 2005. In vivo activity of 11β-hydroxysteroid dehydrogenase type 1 and free fatty acid-induced insulin resistance. Clin. Endocrinol. 63: 442-449.

240. Malhi, H., S. F. Bronk, N. W. Werneburg, and G. J. Gores. 2006. Free fatty acids induce JNK-dependent hepatocyte lipoapoptosis. J Biol Chem. 281: 12093-12101.

241. Marchesini, G., M. Brizi, G. Bianchi, S. Tomassetti, E. Bugianesi, M. Lenzi, A. J. McCullough, S. Natale, G. Forlani, and N. Melchionda. 2001. Nonalcoholic fatty liver disease; a feature of the metabolic syndrome. Diabetes 50: 1844-1850.

242. Marchesini, G., M. Brizi, A. M. Morselli-Labate, G. Bianchi, and E. Bugianesi. 1999. Association of nonalcoholic fatty liver disease with insulin resistance. Am. J. Med. 107: 450-455.

243. Mas, E., M. Danjoux, V. Garcia, S. Carpentier, B. Segui, and T. Levade. 2009. IL-6 deficiency attenuates murine diet-induced non-alcoholic steatohepatitis. PLoS. One. 4: e7929.

244. Masaki, T., S. Chiba, H. Tatsukawa, T. Yasuda, H. Noguchi, M. Seike, and H. Yoshimatsu. 2004. Adiponectin protects LPS-induced liver injury through modulation of TNF-alpha in KK-Ay obese mice. Hepatology 40: 177-184.

245. Masuzaki, H., J. Paterson, H. Shinyama, N. M. Morton, J. J. Mullins, J. R. Seckl, and J. S. Flier. 2001. A Transgenic Model of Visceral Obesity and the Metabolic Syndrome. Science 294: 2166-2170.

246. Masuzaki, H., H. Yamamoto, C. J. Kenyon, J. K. Elmquist, N. M. Morton, J. M. Paterson, H. Shinyama, M. G. F. Sharp, S. Fleming, J. J. Mullins, J. R. Seckl, and J. S. Flier. 2003. Transgenic amplification of glucocorticoid action in adipose tissue causes high blood pressure in mice. J. Clin. Invest. 112: 83-90.

247. Matarese, G. 2000. Leptin and the immune system: how nutritional status influences the immune response. Eur. Cytokine Netw. 11: 7-14.

248. Matsumoto, T., S. Yamasaki, A. Arakawa, K. Abe, H. Abe, K. Kon, S. Kobayashi, and Y. Takasaki. 2007. Exposure to a high total dosage of glucocorticoids produces non-alcoholic steatohepatits. Pathol. Int. 57: 388-389.

249. Matsumoto, T., T. Yoshimine, K. Shimouchi, H. Shiotu, N. Kuwabara, Y. Fukuda, and T. Hoshi. 1992. The liver in systemic lupus erythematosus: pathologic analysis of 52 cases and review of Japanese Autopsy Registry Data. Hum. Pathol. 23: 1151-1158.

250. Matteoni, C. A., Z. M. Younossi, T. Gramlich, N. Boparai, Y. C. Liu, and A. J. McCullough. 1999. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. Gastroenterology 116: 1413-1419.

251. Mayo-Smith, W., C. W. Hayes, B. M. Biller, A. Klibanski, H. Rosenthal, and D. I. Rosenthal. 1989. Body fat distribution measured with CT: correlations in healthy subjects, patients with anorexia nervosa, and patients with Cushing syndrome. Radiology 170: 515-518.

252. McGarry, J. D. and N. F. Brown. 1997. The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. Eur. J Biochem. 244: 1-14.

253. McKay, L. I. and J. A. Cidlowski. 1999. Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. Endocr. Rev. 20: 435-459.

254. McNeilly, A. D., D. P. Macfarlane, E. O'Flaherty, D. E. Livingstone, T. Mitic, K. M. McConnell, S. M. McKenzie, E. Davies, R. M. Reynolds, H. C. Thiesson, O. Skott, B. R. Walker, and R. Andrew. 2010. Bile acids modulate glucocorticoid metabolism and the hypothalamic-pituitary-adrenal axis in obstructive jaundice. J Hepatol. 52: 705-711.

255. Mead, J. R., S. A. Irvine, and D. P. Ramji. 2002. Lipoprotein lipase: structure, function, regulation, and role in disease. J Mol. Med. 80: 753-769.

256. Melgert, B. N., P. Olinga, J. M. Van Der Laan, B. Weert, J. Cho, D. Schuppan, G. M. Groothuis, D. K. Meijer, and K. Poelstra. 2001. Targeting dexamethasone to Kupffer cells: effects on liver inflammation and fibrosis in rats. Hepatology 34: 719-728.

257. Mensenkamp, A. R., L. M. Havekes, J. A. Romijn, and F. Kuipers. 2001. Hepatic steatosis and very low density lipoprotein secretion: the involvement of apolipoprotein E. J Hepatol. 35: 816-822.

258. Mensenkamp, A. R., M. C. Jong, G. H. van, M. J. van Luyn, V. Bloks, R. Havinga, P. J. Voshol, M. H. Hofker, K. W. van Dijk, L. M. Havekes, and F. Kuipers. 1999. Apolipoprotein E participates in the regulation of very low density lipoprotein-triglyceride secretion by the liver. J Biol Chem. 274: 35711-35718.

259. Michailidou, Z., M. D. Jensen, D. A. Dumesic, K. E. Chapman, J. R. Seckl, B. R. Walker, and N. M. Morton. 2007. Omental 11beta-hydroxysteroid dehydrogenase 1 correlates with fat cell size independently of obesity. Obesity. (Silver. Spring) 15: 1155-1163.

260. Miele, L., A. Forgione, T. G. La, V. Vero, C. Cefalo, S. Racco, V. G. Vellone, F. M. Vecchio, G. Gasbarrini, G. L. Rapaccini, M. G. Neuman, and A. Grieco. 2009. Serum levels of hyaluronic acid and tissue metalloproteinase inhibitor-1 combined with age predict the presence of nonalcoholic steatohepatitis in a pilot cohort of subjects with nonalcoholic fatty liver disease. Transl. Res. 154: 194-201.

261. Miles, J. M. and M. D. Jensen. 2005. Counterpoint: Visceral Adiposity Is Not Causally Related to Insulin Resistance. Diabetes Care 28: 2326-2328.

262. Minehira, K., S. G. Young, C. J. Villanueva, L. Yetukuri, M. Oresic, M. K. Hellerstein, R. V. Farese, Jr., J. D. Horton, F. Preitner, B. Thorens, and L. Tappy. 2008. Blocking VLDL secretion causes hepatic steatosis but does not affect peripheral lipid stores or insulin sensitivity in mice. J Lipid Res. 49: 2038-2044.

263. Mittendorfer, B., O. Liem, B. W. Patterson, J. M. Miles, and S. Klein. 2003. What Does the Measurement of Whole-Body Fatty Acid Rate of Appearance in Plasma by Using a Fatty Acid Tracer Really Mean? Diabetes 52: 1641-1648.

264. Miyazaki, M., M. T. Flowers, H. Sampath, K. Chu, C. Otzelberger, X. Liu, and J. M. Ntambi. 2007. Hepatic stearoyl-CoA desaturase-1 deficiency protects mice from carbohydrate-induced adiposity and hepatic steatosis. Cell Metab 6: 484-496.

265. Miyoshi, H., G. I. Shulman, E. J. Peters, M. H. Wolfe, D. Elahi, and R. R. Wolfe. 1988. Hormonal control of substrate cycling in humans. Journal of Clinical Investigation. Vol. 81(5)()(pp 1545-1555), 1988. 1545-1555.

266. Moller, N., J. O. Jorgensen, O. Schmitz, J. Moller, J. Christiansen, K. G. Alberti, and H. Orskov. 1990. Effects of a growth hormone pulse on total and forearm substrate fluxes in humans. Am J Physiol 258: E86-E91.

267. Monetti, M., M. C. Levin, M. J. Watt, M. P. Sajan, S. Marmor, B. K. Hubbard, R. D. Stevens, J. R. Bain, C. B. Newgard, R. V. Farese, Sr., A. L. Hevener, and R. V. Farese, Jr. 2007. Dissociation of hepatic steatosis and insulin resistance in mice overexpressing DGAT in the liver. Cell Metab 6: 69-78.

268. Morrison, W. R. and L. M. Smith. 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. J Lipid Res 5: 600-608.

269. Morton, N. M., M. C. Holmes, C. Fievet, B. Staels, A. Tailleux, J. J. Mullins, and J. R. Seckl. 2001. Improved lipid and lipoprotein profile, hepatic insulin sensitivity, and glucose tolerance in 11β-hydroxysteroid dehydrogenase type 1 null mice. J. Biol. Chem. 276: 41293-41300.

270. Morton, N. M., J. M. Paterson, H. Masuzaki, M. C. Holmes, B. Staels, C. Fievet, B. R. Walker, J. S. Flier, J. J. Mullins, and J. R. Seckl. 2004. Novel adipose tissue-mediated resistance to diet-induced visceral obesity in 11β-hydroxysteroid dehydrogenase type 1 deficient mice. Diabetes 53: 931-938.

271. Mu, Y. P., T. Ogawa, and N. Kawada. 2010. Reversibility of fibrosis, inflammation, and endoplasmic reticulum stress in the liver of rats fed a methionine-choline-deficient diet. Lab Invest 90: 245-256.

272. Nagle, C. A., J. An, M. Shiota, T. P. Torres, G. W. Cline, Z. X. Liu, S. Wang, R. L. Catlin, G. I. Shulman, C. B. Newgard, and R. A. Coleman. 2007. Hepatic overexpression of glycerol-sn-3-phosphate acyltransferase 1 in rats causes insulin resistance. J Biol Chem. 282: 14807-14815.

273. Nakada, M. T., J. M. Stadel, K. S. Poksay, and S. T. Crooke. 1987. Glucocorticoid regulation of beta-adrenergic receptors in 3T3-L1 preadipocytes. Mol. Pharmacol. 31: 377-384.

274. Nakae, D. 1999. Endogenous liver carcinogenesis in the rat. Pathol. Int. 49: 1028-1042.

275. Namikawa, C., Z. Shu-Ping, J. R. Vyselaar, Y. Nozaki, Y. Nemoto, M. Ono, N. Akisawa, T. Saibara, M. Hiroi, H. Enzan, and S. Onishi. 2004. Polymorphisms of microsomal triglyceride transfer protein gene and manganese superoxide dismutase gene in non-alcoholic steatohepatitis. J Hepatol. 40: 781-786.

276. Neschen, S., K. Morino, L. E. Hammond, D. Zhang, Z. X. Liu, A. J. Romanelli, G. W. Cline, R. L. Pongratz, X. M. Zhang, C. S. Choi, R. A. Coleman, and G. I. Shulman. 2005. Prevention of hepatic steatosis and hepatic insulin resistance in mitochondrial acyl-CoA:glycerol-sn-3-phosphate acyltransferase 1 knockout mice. Cell Metab 2: 55-65.

277. Nielsen, S., Z. Guo, C. M. Johnson, D. D. Hensrud, and M. D. Jensen. 2004. Splanchnic lipolysis in human obesity. J. Clin. Invest. 113: 1582-1588.

278. Norrheim, L., H. Sorensen, K. Gautvik, J. Bremer, and O. Spydevold. 1990. Synergistic actions of tetradecylthioacetic acid (TTA) and dexamethasone on induction of the peroxisomal beta-oxidation and on growth inhibition of Morris hepatoma cells. Both effects are counteracted by insulin. Biochim. Biophys. Acta 1051: 319-323.

279. Oishi, Y., Z. W. Fu, Y. Ohnuki, H. Kato, and T. Noguchi. 2002. Molecular basis of the alteration in skin collagen metabolism in response to in vivo dexamethasone treatment: effects on the synthesis of collagen type I and III, collagenase, and tissue inhibitors of metalloproteinases. Br. J Dermatol. 147: 859-868.

280. Okamoto, K., K. Mimura, Y. Murawaki, and I. Yuasa. 2005. Association of functional gene polymorphisms of matrix metalloproteinase (MMP)-1, MMP-3 and MMP-9 with the progression of chronic liver disease. J Gastroenterol. Hepatol. 20: 1102-1108.

281. Okumura, K., K. Ikejima, K. Kon, W. Abe, S. Yamashina, N. Enomoto, Y. Takei, and N. Sato. 2006. Exacerbation of dietary steatohepatitis and fibrosis in obese, diabetic KK-Ay mice. Hepatology Research 36: 217-228.

282. Olofsson, S. O. and J. Boren. 2005. Apolipoprotein B: a clinically important apolipoprotein which assembles atherogenic lipoproteins and promotes the development of atherosclerosis. J Intern. Med. 258: 395-410.

283. Ong, J., Z. M. Younossi, V. Reddy, L. L. Price, T. Gramlich, J. Mayes, and N. Boparai. 2001. Cryptogenic cirrhosis and posttransplantation nonalcoholic fatty liver disease. Liver Transpl. 7: 797-801.

284. Ong, J. M. and P. A. Kern. 1989. Effect of feeding and obesity on lipoprotein lipase activity, immunoreactive protein, and messenger RNA levels in human adipose tissue. J Clin Invest 84: 305-311.

285. Ong, J. M., R. B. Simsolo, B. Saffari, and P. A. Kern. 1992. The regulation of lipoprotein lipase gene expression by dexamethasone in isolated rat adipocytes. Endocrinology 130: 2310-2316.

286. Oppermann, U. C., K. J. Netter, and E. Maser. 1995. Cloning and primary structure of murine 11 beta-hydroxysteroid dehydrogenase/microsomal carbonyl reductase. Eur. J Biochem. 227: 202-208.

287. Ottosson, M., P. Lonnroth, P. Bjorntorp, and S. Eden. 2000. Effects of cortisol and growth hormone on lipolysis in human adipose tissue. J Clin Endocrinol Metab 85: 799-803.

288. Ottosson, M., P. Marin, K. Karason, A. Elander, and P. Bjorntorp. 1995. Blockade of the glucocorticoid receptor with RU 486: effects in vitro and in vivo on human adipose tissue lipoprotein lipase activity. Obes. Res. 3: 233-240.

289. Oz, H. S., H. J. Im, T. S. Chen, W. J. de Villiers, and C. J. McClain. 2006. Glutathione-enhancing agents protect against steatohepatitis in a dietary model. J Biochem. Mol. Toxicol. 20: 39-47.

290. Ozturk, H., H. Eken, H. Ozturk, and H. Buyukbayram. 2006. Effects of dexamethasone on small bowel and kidney oxidative stress and histological alterations in bile duct-ligated rats. Pediatr. Surg. Int. 22: 709-718.

291. Pan, X., F. N. Hussain, J. Iqbal, M. H. Feuerman, and M. M. Hussain. 2007. Inhibiting proteasomal degradation of microsomal triglyceride transfer protein prevents CCl4-induced steatosis. J Biol Chem. 282: 17078-17089.

292. Pankow, J. S., B. B. Duncan, M. I. Schmidt, C. M. Ballantyne, D. J. Couper, R. C. Hoogeveen, and S. H. Golden. 2004. Fasting plasma free fatty acids and risk of type 2 diabetes: The ARIC Study. Circulation 109: E120.

293. Paterson, J. M., Holmes, M. C., Morton, N. M., Seckl, J. R., and Mullins, J. J. Liver-specific over-expression of 11β-HSD1 in transgenic mice produces insulin resistance, hyperlipidaemia and fatty liver. Proceedings of Endo 2002 , P2-319. 2002.
Ref Type: Abstract

294. Paterson, J. M., N. M. Morton, C. Fievet, C. J. Kenyon, M. C. Holmes, B. Staels, J. R. Seckl, and J. J. Mullins. 2004. Metabolic syndrome without obesity: hepatic overexpression of 11β-hydroxysteroid dehydrogenase type 1 in transgenic mice. Proceedings of the National Academy of Sciences of the United States of America 101: 7088-7093.

295. Patterson, B. W., G. Zhao, N. Elias, D. L. Hachey, and S. Klein. 1999. Validation of a new procedure to determine plasma fatty acid concentration and isotopic enrichment. J Lipid Res. 40: 2118-2124.

296. Patterson, B. W., G. Zhao, and S. Klein. 1998. Improved accuracy and precision of gas chromatography/mass spectrometry measurements for metabolic tracers. Metabolism 47: 706-712.

297. Pearlman, F. C. and R. T. Lee. 1974. Detection and measurement of total bilirubin in serum, with use of surfactants as solubilizing agents. Clin Chem. 20: 447-453.

298. Perkins, N. D. 2007. Integrating cell-signalling pathways with NF-kappaB and IKK function. Nat. Rev. Mol. Cell Biol 8: 49-62.

299. Pernet, A., D. G. Johnston, V. Hammond, H. Orskov, and K. G. Alberti. 1986. Interactions of stress hormones on lipid and carbohydrate metabolism in man with partial insulin deficiency. Eur. J Clin Invest 16: 310-315.

300. Peters, R. L., T. Gay, and T. B. Reynolds. 1975. Post-jejunoileal-bypass hepatic disease. Its similarity to alcoholic hepatic disease. Am J Clin. Pathol. 63: 318-331.

301. Petersen, K. F. and G. I. Shulman. 2002. Cellular mechanism of insulin resistance in skeletal muscle. J R. Soc. Med. 95 Suppl 42: 8-13.

302. Petrosillo, G., P. Portincasa, I. Grattagliano, G. Casanova, M. Matera, F. M. Ruggiero, D. Ferri, and G. Paradies. 2007. Mitochondrial dysfunction in rat with nonalcoholic fatty liver Involvement of complex I, reactive oxygen species and cardiolipin. Biochim. Biophys. Acta 1767: 1260-1267.

303. Phung, N., N. Pera, G. Farrell, I. Leclercq, J. Y. Hou, and J. George. 2009. Pro-oxidant-mediated hepatic fibrosis and effects of antioxidant intervention in murine dietary steatohepatitis. Int. J Mol. Med. 24: 171-180.

304. Pinzani, M., L. Gesualdo, G. M. Sabbah, and H. E. Abboud. 1989. Effects of platelet-derived growth factor and other polypeptide mitogens on DNA synthesis and growth of cultured rat liver fat-storing cells. J Clin Invest 84: 1786-1793.

305. Pittner, R. A., R. Fears, and D. N. Brindley. 1985. Interactions of insulin, glucagon and dexamethasone in controlling the activity of glycerol phosphate acyltransferase and the activity and subcellular distribution of phosphatidate phosphohydrolase in cultured rat hepatocytes. Biochem. J 230: 525-534.

306. Postic, C. and J. Girard. 2008. Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. J Clin Invest 118: 829-838.

307. Raabe, M., M. M. Veniant, M. A. Sullivan, C. H. Zlot, J. Bjorkegren, L. B. Nielsen, J. S. Wong, R. L. Hamilton, and S. G. Young. 1999. Analysis of the role of microsomal triglyceride transfer protein in the liver of tissue-specific knockout mice. J Clin Invest 103: 1287-1298.

308. Rahman, S. M., J. M. Schroeder-Gloeckler, R. C. Janssen, H. Jiang, I. Qadri, K. N. Maclean, and J. E. Friedman. 2007. CCAAT/enhancing binding protein beta deletion in mice attenuates inflammation, endoplasmic reticulum stress, and lipid accumulation in diet-induced nonalcoholic steatohepatitis. Hepatology 45: 1108-1117.

309. Randle, P. J., P. B. Garland, C. N. Hales, and E. A. Newsholme. 1963. The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. Lancet 1: 785-789.

310. Rask, E., T. Olsson, S. Soderberg, R. Andrew, D. E. W. Livingstone, O. Johnson, and B. R. Walker. 2001. Tissue-specific dysregulation of cortisol metabolism in human obesity. J. Clin. Endocrinol. Metab. 86: 1418-1421.

311. Ratziu, V., P. Giral, F. Charlotte, E. Bruckert, V. Thibault, I. Theodorou, L. Khalil, G. Turpin, P. Opolon, and T. Poynard. 2000. Liver fibrosis in overweight patients. Gastroenterology 118: 1117-1123.

312. Raubenheimer, P. J., M. J. Nyirenda, and B. R. Walker. 2006. A choline-deficient diet exacerbates fatty liver but attenuates insulin resistance and glucose intolerance in mice fed a high-fat diet. Diabetes 55: 2015-2020.

313. Reaven, G. M. and B. B. Hoffman. 1987. A role for insulin in the aetiology and course of hypertension. Lancet ii: 435-436.

314. Rebuffe-Scrive, M., M. Bronnegard, A. Nilsson, J. Eldh, J. A. Gustafsson, and P. Bjorntorp. 1990. Steroid hormone receptors in human adipose tissues. J Clin Endocrinol Metab 71: 1215-1219.

315. Rebuffe-Scrive, M., M. Krotkiewski, J. Elfverson, and P. Bjorntorp. 1988. Muscle and adipose tissue morphology and metabolism in Cushing's syndrome. J Clin Endocrinol Metab 67: 1122-1128.

316. Rebuffe-Scrive, M., U. A. Walsh, B. McEwen, and J. Rodin. 1992. Effect of chronic stress and exogenous glucocorticoids on regional fat distribution and metabolism. Physiol Behav. 52: 583-590.

317. Reddy, J. K. and M. S. Rao. 2006. Lipid metabolism and liver inflammation. II. Fatty liver disease and fatty acid oxidation. Am J Physiol Gastrointest Liver Physiol 290: G852-G858.

318. Reid, B. N., G. P. Ables, O. A. Otlivanchik, G. Schoiswohl, R. Zechner, W. S. Blaner, I. J. Goldberg, R. F. Schwabe, S. C. Chua, Jr., and L. S. Huang. 2008. Hepatic Overexpression of Hormone-sensitive Lipase and Adipose Triglyceride Lipase Promotes Fatty Acid Oxidation, Stimulates Direct Release of Free Fatty Acids, and Ameliorates Steatosis. J. Biol. Chem. 283: 13087-13099.

319. Rhen, T. and J. A. Cidlowski. 2005. Antiinflammatory action of glucocorticoids--new mechanisms for old drugs. N. Engl. J Med. 353: 1711-1723.

320. Rinella, M. E., M. S. Elias, R. R. Smolak, T. Fu, J. Borensztajn, and R. M. Green. 2008. Mechanisms of hepatic steatosis in mice fed a lipogenic methionine choline-deficient diet. J Lipid Res. 49: 1068-1076.

321. Rinella, M. E. and R. M. Green. 2004. The methionine-choline deficient dietary model of steatohepatitis does not exhibit insulin resistance. Journal of Hepatology 40: 47-51.

322. Rizki, G., L. Arnaboldi, B. Gabrielli, J. Yan, G. S. Lee, R. K. Ng, S. M. Turner, T. M. Badger, R. E. Pitas, and J. J. Maher. 2006. Mice fed a lipogenic methionine-choline-deficient diet develop hypermetabolism coincident with hepatic suppression of SCD-1. J Lipid Res. 47: 2280-2290.

323. Robertson, G., I. Leclercq, and G. C. Farrell. 2001. Nonalcoholic Steatosis and Steatohepatitis: II. Cytochrome P-450 enzymes and oxidative stress. Am J Physiol Gastrointest Liver Physiol 281: G1135-G1139.

324. Rockall, A. G., S. A. Sohaib, D. Evans, G. Kaltsas, A. M. Isidori, J. P. Monson, G. M. Besser, A. B. Grossman, and R. H. Reznek. 2003. Hepatic steatosis in Cushing's syndrome: a radiological assessment using computed tomography. Eur. J Endocrinol 149: 543-548.

325. Rockey, D. C., J. K. Boyles, G. Gabbiani, and S. L. Friedman. 1992. Rat hepatic lipocytes express smooth muscle actin upon activation in vivo and in culture. J Submicrosc. Cytol. Pathol. 24: 193-203.

326. Roeb, E., L. Graeve, R. Hoffmann, K. Decker, D. R. Edwards, and P. C. Heinrich. 1993. Regulation of tissue inhibitor of metalloproteinases-1 gene expression by cytokines and dexamethasone in rat hepatocyte primary cultures. Hepatology 18: 1437-1442.

327. Romeo, S., I. Huang-Doran, M. G. Baroni, and A. Kotronen. 2010. Unravelling the pathogenesis of fatty liver disease: patatin-like phospholipase domain-containing 3 protein. Curr. Opin. Lipidol. 21: 247-252.

328. Romeo, S., J. Kozlitina, C. Xing, A. Pertsemlidis, D. Cox, L. A. Pennacchio, E. Boerwinkle, J. C. Cohen, and H. H. Hobbs. 2008. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nat. Genet. 40: 1461-1465.

329. Romestaing, C., M. A. Piquet, D. Letexier, B. Rey, A. Mourier, S. Servais, M. Belouze, V. Rouleau, M. Dautresme, I. Ollivier, R. Favier, M. Rigoulet, C. Duchamp, and B. Sibille. 2008. Mitochondrial adaptations to steatohepatitis induced by a methionine- and choline-deficient diet. Am J Physiol Endocrinol Metab 294: E110-E119.

330. Rosen, T., I. Bosaeus, J. Tolli, G. Lindstedt, and B. A. Bengtsson. 1993. Increased body fat mass and decreased extracellular fluid volume in adults with growth hormone deficiency. Clin. Endocrinol (Oxf) 38: 63-71.

331. Rosenstock, J., S. Banarer, V. A. Fonseca, S. E. Inzucchi, W. Sun, W. Yao, G. Hollis, R. Flores, R. Levy, W. V. Williams, J. R. Seckl, and R. Huber. 2010. The 11-beta-hydroxysteroid dehydrogenase type 1 inhibitor INCB13739 improves hyperglycemia in patients with type 2 diabetes inadequately controlled by metformin monotherapy. Diabetes Care 33: 1516-1522.

332. Sahai, A., P. Malladi, H. Melin-Aldana, R. M. Green, and P. F. Whitington. 2004a. Upregulation of osteopontin expression is involved in the development of nonalcoholic steatohepatitis in a dietary murine model. Am J Physiol Gastrointest Liver Physiol 287: G264-G273.

333. Sahai, A., P. Malladi, X. Pan, R. Paul, H. Melin-Aldana, R. M. Green, and P. F. Whitington. 2004b. Obese and diabetic db/db mice develop marked liver fibrosis in a model of nonalcoholic steatohepatitis: role of short-form leptin receptors and osteopontin. Am J Physiol Gastrointest Liver Physiol 287: G1035-G1043.

334. Saito, J. M., M. K. Bostick, C. B. Campe, J. Xu, and J. J. Maher. 2003. Infiltrating neutrophils in bile duct-ligated livers do not promote hepatic fibrosis. Hepatol. Res. 25: 180-191.

335. Sakaida, I., Y. Matsumura, S. Akiyama, K. Hayashi, A. Ishige, and K. Okita. 1998. Herbal medicine Sho-saiko-to (TJ-9) prevents liver fibrosis and enzyme-altered lesions in rat liver cirrhosis induced by a choline-deficient L-amino acid-defined diet. J Hepatol. 28: 298-306.

336. Samra, J. S., M. L. Clark, S. M. Humphreys, I. A. MacDonald, P. A. Bannister, Frayn, and KN. 1998. Effects of physiological hypercortisolemia on the regulation of lipolysis in subcutaneous adipose tissue. J. Clin. Endocrinol. Metab. 83: 626-631.

337. Samra, J. S., M. L. Clark, S. M. Humphreys, I. A. MacDonald, D. R. Matthews, and K. N. Frayn. 1996a. Effects of morning rise in cortisol concentration on regulation of lipolysis in subcutaneous adipose tissue. Am J Physiol 271: E996-1002.

338. Samra, J. S., E. J. Simpson, M. L. Clark, C. D. Forster, S. M. Humphreys, I. A. MacDonald, and K. N. Frayn. 1996b. Effects of epinephrine infusion on adipose tissue: interactions between blood flow and lipid metabolism. Am J Physiol Endocrinol Metab 271: E834-E839.

339. Sandeep, T. C., R. Andrew, N. Z. M. Homer, R. C. Andrews, K. Smith, and B. R. Walker. 2005. Increased in vivo regeneration of cortisol in adipose tissue in human obesity and effects of the 11β-hydroxysteroid dehydrogenase type 1 inhibitor carbenoxolone. Diabetes 54: 872-879.

340. Santomauro, A. T., G. Boden, M. E. Silva, D. M. Rocha, R. F. Santos, M. J. Ursich, P. G. Strassmann, and B. L. Wajchenberg. 1999. Overnight lowering of free fatty acids with Acipimox improves insulin resistance and glucose tolerance in obese diabetic and nondiabetic subjects. Diabetes 48: 1836-1841.

341. Sanyal, A. J. 2002. AGA technical review on nonalcoholic fatty liver disease. Gastroenterology 123: 1705-1725.

342. Sanyal, A. J., C. Campbell-Sargent, F. Mirshahi, W. B. Rizzo, M. J. Contos, R. K. Sterling, V. A. Luketic, M. L. Shiffman, and J. N. Clore. 2001. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. Gastroenterology 120: 1183-1192.

343. Saruc, M., H. Yuceyar, S. Ayhan, N. Turkel, I. Tuzcuoglu, and M. Can. 2003. The association of dehydroepiandrosterone, obesity, waist-hip ratio and insulin resistance with fatty liver in postmenopausal women--a hyperinsulinemic euglycemic insulin clamp study. Hepatogastroenterology 50: 771-774.

344. Sato, W., Y. Horie, E. Kataoka, S. Ohshima, T. Dohmen, M. Iizuka, J. Sasaki, T. Sasaki, K. Hamada, H. Kishimoto, A. Suzuki, and S. Watanabe. 2006. Hepatic gene expression in hepatocyte-specific Pten deficient mice showing steatohepatitis without ethanol challenge. Hepatol. Res. 34: 256-265.

345. Saunders, J., S. E. Hall, and P. H. Sonksen. 1980. Glucose and free fatty acid turnover in Cushing's syndrome. J Endocrinol Invest 3: 309-311.

346. Schade, D. S., R. P. Eaton, and J. Standefer. 1978. Modulation of basal ketone body concentration by cortisol in diabetic man. J Clin. Endocrinol. Metab 47: 519-528.

347. Schattenberg, J. M., R. Singh, R. M. Rigoli, and M. J. Czaja. 2005. Hepatocyte CYP2E1 overexpression and steatohepatitis lead to impaired hepatic insulin signaling. J. Biol. Chem. 279: 23916-23924.

348. Schattenberg, J. M., R. Singh, Y. Wang, J. H. Lefkowitch, R. M. Rigoli, P. E. Scherer, and M. J. Czaja. 2006. JNK1 but not JNK2 promotes the development of steatohepatitis in mice. Hepatology 43: 163-172.

349. Schoenborn, V., I. M. Heid, C. Vollmert, A. Lingenhel, T. D. Adams, P. N. Hopkins, T. Illig, R. Zimmermann, R. Zechner, S. C. Hunt, and F. Kronenberg. 2006. The ATGL gene is associated with free fatty acids, triglycerides, and type 2 diabetes. Diabetes 55: 1270-1275.

350. Schoenheimer, R. and D. Rittenberg. 1935. Deuterium as an indicator in the study of intermediary metabolism. Science 82: 156-157.

351. Schonfeld, G., B. W. Patterson, D. A. Yablonskiy, T. S. Tanoli, M. Averna, N. Elias, P. Yue, and J. Ackerman. 2003. Fatty liver in familial hypobetalipoproteinemia: triglyceride assembly into VLDL particles is affected by the extent of hepatic steatosis. J Lipid Res. 44: 470-478.

352. Schonfeld, G., P. Yue, X. Lin, and Z. Chen. 2008. Fatty liver and insulin resistance: not always linked. Trans. Am Clin Climatol. Assoc. 119: 217-223.

353. Schoonjans, K., B. Staels, and J. Auwerx. 1996. Role of the peroxisome proliferator-activated receptor (PPAR) in mediating the effects of fibrates and fatty acids on gene expression. J Lipid Res. 37: 907-925.

354. Schuppan, D. and N. H. Afdhal. 2008. Liver cirrhosis. Lancet 371: 838-851.

355. Schuppan, D., M. Ruehl, R. Somasundaram, and E. G. Hahn. 2001. Matrix as a modulator of hepatic fibrogenesis. Semin. Liver Dis. 21: 351-372.

356. Scow, R. O., E. M. Wagner, and A. CARDEZA. 1957. Effect of hypophysectomy on the insulin requirement and response to fasting of totally pancreatectomized rats. Endocrinology 61: 380-391.

357. Scribner, K. B., D. B. Pawlak, C. M. Aubin, J. A. Majzoub, and D. S. Ludwig. 2008. Long-term effects of dietary glycemic index on adiposity, energy metabolism, and physical activity in mice. Am J Physiol Endocrinol Metab 295: E1126-E1131.

358. Seckl, J. R. and B. R. Walker. 2001. 11β-Hydroxysteroid dehydrogenase type 1 - a tissue-specific amplifier of glucocorticoid action. Endocrinology 142: 1371-1376.

359. Seppala-Lindroos, A., S. Vehkavaara, A. M. Hakkinen, T. Goto, J. Westerbacka, A. Sovijarvi, Halavaara.J, and H. Yki-Jarvinen. 2002. Fat accumulation in the liver is associated with defects in insulin suppression of glucose production and serum free fatty acids independent of obesity in normal men. J. Clin. Endocrinol. Metab. 87: 3023-3028.

360. Shadid, S. and M. D. Jensen. 2003. Effects of Pioglitazone Versus Diet and Exercise on Metabolic Health and Fat Distribution in Upper Body Obesity. Diabetes Care 26: 3148-3152.

361. Short, K. R., J. Nygren, M. L. Bigelow, and K. S. Nair. 2004. Effect of Short-Term Prednisone Use on Blood Flow, Muscle Protein Metabolism, and Function. J Clin Endocrinol Metab 89: 6198-6207.

362. Sidossis, L. S., C. A. Stuart, G. I. Shulman, G. D. Lopaschuk, and R. R. Wolfe. 1996. Glucose plus insulin regulate fat oxidation by controlling the rate of fatty acid entry into the mitochondria. J Clin Invest 98: 2244-2250.

363. Slavin, B. G., J. M. Ong, and P. A. Kern. 1994. Hormonal regulation of hormone-sensitive lipase activity and mRNA levels in isolated rat adipocytes. J. Lipid Res. 35: 1535-1541.

364. Soumano, K., S. Desbiens, R. Rabelo, E. Bakopanos, A. Camirand, and J. E. Silva. 2000. Glucocorticoids inhibit the transcriptional response of the uncoupling protein-1 gene to adrenergic stimulation in a brown adipose cell line. Mol. Cell Endocrinol 165: 7-15.

365. Spaniol, M., H. Brooks, L. Auer, A. Zimmermann, M. Solioz, B. Stieger, and S. Krahenbuhl. 2001. Development and characterization of an animal model of carnitine deficiency. Eur. J Biochem. 268: 1876-1887.

366. Spector, A. A. 1975. Fatty acid binding to plasma albumin. J Lipid Res. 16: 165-179.

367. Stanhope, K. L. and P. J. Havel. 2008. Endocrine and metabolic effects of consuming beverages sweetened with fructose, glucose, sucrose, or high-fructose corn syrup. Am J Clin Nutr 88: 1733S-1737S.

368. Steele, R. 1959. Influences of glucose loading and of injected insulin on hepatic glucose output. Ann. N. Y. Acad. Sci 82: 420-430.

369. Stewart, P. M., A. Boulton, S. Kumar, P. M. S. Clark, and C. H. L. Shackleton. 1999. Cortisol metabolism in human obesity: impaired cortisone - cortisol conversion in subjects with central adiposity. J. Clin. Endocrinol. Metab. 84: 1022-1027.

370. Stiles, B., Y. Wang, A. Stahl, S. Bassilian, W. P. Lee, Y. J. Kim, R. Sherwin, S. Devaskar, R. Lesche, M. A. Magnuson, and H. Wu. 2004. Liver-specific deletion of negative regulator Pten results in fatty liver and insulin hypersensitivity [corrected]. Proc. Natl. Acad. Sci. U. S. A 101: 2082-2087.

371. Stimson, R. H., J. Andersson, R. Andrew, D. N. Redhead, F. Karpe, P. C. Hayes, T. Olsson, and B. R. Walker. 2009. Cortisol release from adipose tissue by 11beta-hydroxysteroid dehydrogenase type 1 in humans. Diabetes 58: 46-53.

372. Stimson, R. H., Andrew, R., McAvoy, N., Tripathi, D., Hayes, P., and Walker, B. R. Hepatic vein cannulation and stable isotope tracer infusion reveals that liver cortisol regeneration by 11ß-HSD1 is sustained in obese men with type 2 diabetes mellitus, providing a target for enzyme inhibition. Endocrine Abstracts 21, 385. 2010.
Ref Type: Abstract

373. Stimson, R. H., A. M. Johnstone, N. Z. Homer, D. J. Wake, N. M. Morton, R. Andrew, G. E. Lobley, and B. R. Walker. 2007. Dietary macronutrient content alters cortisol metabolism independently of body weight changes in obese men. J Clin. Endocrinol Metab 92: 4480-4484.

374. Stimson, R. H. and B. R. Walker. 2007. Glucocorticoids and 11beta-hydroxysteroid dehydrogenase type 1 in obesity and the metabolic syndrome. Minerva Endocrinol 32: 141-159.

375. Stone, S. J., H. M. Myers, S. M. Watkins, B. E. Brown, K. R. Feingold, P. M. Elias, and R. V. Farese, Jr. 2004. Lipopenia and skin barrier abnormalities in DGAT2-deficient mice. J Biol Chem. 279: 11767-11776.

376. Strawford, A., F. Antelo, M. Christiansen, and M. K. Hellerstein. 2004. Adipose tissue triglyceride turnover, de novo lipogenesis, and cell proliferation in humans measured with 2H2O. Am J Physiol Endocrinol Metab 286: E577-E588.

377. Sugimoto, H., K. Okada, J. Shoda, E. Warabi, K. Ishige, T. Ueda, K. Taguchi, T. Yanagawa, A. Nakahara, I. Hyodo, T. Ishii, and M. Yamamoto. 2010. Deletion of nuclear factor-E2-related factor-2 leads to rapid onset and progression of nutritional steatohepatitis in mice. Am J Physiol Gastrointest Liver Physiol 298: G283-G294.

378. Sumida, C. 1995. Fatty acids: ancestral ligands and modern co-regulators of the steroid hormone receptor cell signalling pathway. Prostaglandins Leukotrienes and Essential Fatty Acids 52: 137-144.

379. Svedberg, J., P. Bjorntorp, U. Smith, and P. Lonnroth. 1990. Free-fatty acid inhibition of insulin binding, degradation, and action in isolated rat hepatocytes. Diabetes 39: 570-574.

380. Svegliati-Baroni, G., C. Candelaresi, S. Saccomanno, G. Ferretti, T. Bachetti, M. Marzioni, M. S. De, L. Nobili, R. Salzano, A. Omenetti, D. Pacetti, S. Sigmund, A. Benedetti, and A. Casini. 2006. A model of insulin resistance and nonalcoholic steatohepatitis in rats: role of peroxisome proliferator-activated receptor-alpha and n-3 polyunsaturated fatty acid treatment on liver injury. Am J Pathol. 169: 846-860.

381. Swain, M. G., C. Appleyard, J. Wallace, H. Wong, and T. Le. 1999. Endogenous glucocorticoids released during acute toxic liver injury enhance hepatic IL-10 synthesis and release. Am. J. Physiol. 276: G199-G205.

382. Syn, W. K., S. S. Choi, and A. M. Diehl. 2009. Apoptosis and cytokines in non-alcoholic steatohepatitis. Clin Liver Dis. 13: 565-580.

383. Szczepaniak, L. S., P. Nurenberg, D. Leonard, J. D. Browning, J. S. Reingold, S. Grundy, H. H. Hobbs, and R. L. Dobbins. 2005. Magnetic resonance spectroscopy to measure hepatic triglyceride content: prevalence of hepatic steatosis in the general population. Am J Physiol Endocrinol. Metab 288: E462-E468.

384. Tahan, V., D. Yavuz, N. Imeryuz, E. Avsar, and N. Tozun. 2004. Oral glucose tolerance deteriorates in rats fed with methionine choline deficient diet. J Hepatol. 41: 352.

385. Targher, G., L. Bertolini, S. Rodella, G. Zoppini, L. Zenari, and G. Falezza. 2006. Associations between liver histology and cortisol secretion in subjects with nonalcoholic fatty liver disease. Clin. Endocrinol. (Oxf) 64: 337-341.

386. Targher, G., L. Bertolini, G. Zoppini, L. Zenari, and G. Falezza. 2005. Relationship of non-alcoholic hepatic steatosis to cortisol secretion in diet-controlled Type 2 diabetic patients. Diabet. Med. 22: 1146-1150.

387. Taskinen, M. R., E. A. Nikkila, R. Pelkonen, and T. Sane. 1983. Plasma lipoproteins, lipolytic enzymes, and very low density lipoprotein triglyceride turnover in Cushing's syndrome. J Clin Endocrinol Metab 57: 619-626.

388. Tataranni, P. A., D. E. Larson, S. Snitker, J. B. Young, J. P. Flatt, and E. Ravussin. 1996. Effects of glucocorticoids on energy metabolism and food intake in humans. Am J Physiol 271: E317-E325.

389. Teli, M. R., O. F. James, A. D. Burt, M. K. Bennett, and C. P. Day. 1995. The natural history of nonalcoholic fatty liver: a follow-up study. Hepatology 22: 1714-1719.

390. Thieringer, R., C. B. Le Grand, L. Carbin, T. Q. Cai, B. Wong, S. D. Wright, and A. Hermanowski-Vosatka. 2001. 11 Beta-hydroxysteroid dehydrogenase type 1 is induced in human monocytes upon differentiation to macrophages. J Immunol. 167: 30-35.

391. Tietge, U. J. F., A. Bakillah, C. Maugeais, K. Tsukamoto, M. Hussain, and D. J. Rader. 1999. Hepatic overexpression of microsomal triglyceride transfer protein (MTP) results in increased in vivo secretion of VLDL triglycerides and apolipoprotein B. J. Lipid Res. 40: 2134-2139.

392. Timmermans, P. B., P. C. Wong, A. T. Chiu, W. F. Herblin, P. Benfield, D. J. Carini, R. J. Lee, R. R. Wexler, J. A. Saye, and R. D. Smith. 1993. Angiotensin II receptors and angiotensin II receptor antagonists. Pharmacol. Rev. 45: 205-251.

393. Toda, K., K. Takeda, S. Akira, T. Saibara, T. Okada, S. Onishi, and Y. Shizuta. 2001. Alternations in hepatic expression of fatty-acid metabolizing enzymes in ArKO mice and their reversal by the treatment with 17beta-estradiol or a peroxisome proliferator. J Steroid Biochem. Mol. Biol 79: 11-17.

394. Tolman, K. G., V. Fonseca, A. Dalpiaz, and M. H. Tan. 2007. Spectrum of liver disease in type 2 diabetes and management of patients with diabetes and liver disease. Diabetes Care 30: 734-743.

395. Tomita, K., G. Tamiya, S. Ando, K. Ohsumi, T. Chiyo, A. Mizutani, N. Kitamura, K. Toda, T. Kaneko, Y. Horie, J. Y. Han, S. Kato, M. Shimoda, Y. Oike, M. Tomizawa, S. Makino, T. Ohkura, H. Saito, N. Kumagai, H. Nagata, H. Ishii, and T. Hibi. 2006. Tumour necrosis factor alpha signalling through activation of Kupffer cells plays an essential role in liver fibrosis of non-alcoholic steatohepatitis in mice. Gut 55: 415-424.

396. Tomlinson, J. W., J. Moore, M. S. Cooper, I. Bujalska, M. Shahmanesh, C. Burt, A. Strain, M. Hewison, and P. M. Stewart. 2001. Regulation of expression of 11β-hydroxysteroid dehydrogenase type 1 in adipose tissue: tissue-specific induction by cytokines. Endocrinology 142: 1982-1989.

397. Tomlinson, J. W., B. Sinha, I. Bujalska, M. Hewison, and P. M. Stewart. 2002. Expression of 11beta-hydroxysteroid dehydrogenase type 1 in adipose tissue is not increased in human obesity. J. Clin. Endocrinol. Metab. 87: 5630-5635.

398. Tomlinson, J. W., E. A. Walker, I. J. Bujalska, N. Draper, G. G. Lavery, M. S. Cooper, M. Hewison, and P. M. Stewart. 2004. 11beta-Hydroxysteroid dehydrogenase type 1: A tissue-specific regulator of glucocorticoid response. Endocr. Rev. 25: 831-866.

399. Tsukamoto, K., C. Maugeais, J. M. Glick, and D. J. Rader. 2000. Markedly increased secretion of VLDL triglycerides induced by gene transfer of apolipoprotein E isoforms in apoE-deficient mice. J Lipid Res. 41: 253-259.

400. Udden, J., P. Bjorntorp, P. Arner, B. Barkeling, L. Meurling, and S. Rossner. 2003. Effects of glucocorticoids on leptin levels and eating behaviour in women. J Intern. Med. 253: 225-231.

401. Uno, M., S. Kurita, H. Misu, H. Ando, T. Ota, N. Matsuzawa-Nagata, Y. Kita, S. Nabemoto, H. Akahori, Y. Zen, Y. Nakanuma, S. Kaneko, and T. Takamura. 2008. Tranilast, an antifibrogenic agent, ameliorates a dietary rat model of nonalcoholic steatohepatitis. Hepatology 48: 109-118.

402. van Hall G., A. Steensberg, M. Sacchetti, C. Fischer, C. Keller, P. Schjerling, N. Hiscock, K. Moller, B. Saltin, M. A. Febbraio, and B. K. Pedersen. 2003. Interleukin-6 stimulates lipolysis and fat oxidation in humans. J Clin Endocrinol Metab 88: 3005-3010.

403. Vetelainen, R., V. A. van, and T. M. van Gulik. 2007. Essential pathogenic and metabolic differences in steatosis induced by choline or methione-choline deficient diets in a rat model. J Gastroenterol. Hepatol. 22: 1526-1533.

404. Vidali, M., M. F. Tripodi, A. Ivaldi, R. Zampino, G. Occhino, L. Restivo, S. Sutti, A. Marrone, G. Ruggiero, E. Albano, and L. E. Adinolfi. 2008. Interplay between oxidative stress and hepatic steatosis in the progression of chronic hepatitis C. J Hepatol. 48: 399-406.

405. Villena, J. A., S. Roy, E. Sarkadi-Nagy, K. H. Kim, and H. S. Sul. 2004. Desnutrin, an adipocyte gene encoding a novel patatin domain-containing protein, is induced by fasting and glucocorticoids: ectopic expression of desnutrin increases triglyceride hydrolysis. J Biol. Chem. 279: 47066-47075.

406. Voshol, P. J., G. Haemmerle, D. M. Ouwens, R. Zimmermann, R. Zechner, B. Teusink, J. A. Maassen, L. M. Havekes, and J. A. Romijn. 2003. Increased hepatic insulin sensitivity together with decreased hepatic triglyceride stores in hormone-sensitive lipase-deficient mice. Endocrinology 144: 3456-3462.

407. Wake, D. J., N. Z. Homer, R. Andrew, and B. R. Walker. 2006. Acute in vivo regulation of 11beta-hydroxysteroid dehydrogenase type 1 activity by insulin and intralipid infusions in humans. J Clin. Endocrinol Metab 91: 4682-4688.

408. Wake, D. J., E. Rask, D. E. W. Livingstone, S. Soderberg, T. Olsson, and B. R. Walker. 2003. Local and systemic impact of transcriptional upregulation of 11β-hydroxysteroid dehydrogenase type 1 in adipose tissue in human obesity. J. Clin. Endocrinol. Metab. 88: 2983-2988.

409. Walkey, C. J., L. Yu, L. B. Agellon, and D. E. Vance. 1998. Biochemical and Evolutionary Significance of Phospholipid Methylation. J. Biol. Chem. 273: 27043-27046.

410. Wang, C. N., R. S. McLeod, Z. Yao, and D. N. Brindley. 1995. Effects of Dexamethasone on the Synthesis, Degradation, and Secretion of Apolipoprotein B in Cultured Rat Hepatocytes. Arterioscler Thromb Vasc Biol 15: 1481-1491.

411. Wang, Y., V. B. Jones, S. Urs, S. Kim, M. Soltani-Bejnood, N. Quigley, Y. R. Heo, M. Standridge, B. Andersen, M. Dhar, R. Joshi, P. Wortman, J. W. Taylor, J. Chun, M. Leuze, K. Claycombe, A. M. Saxton, and N. Moustaid-Moussa. 2004. The human fatty acid synthase gene and de novo lipogenesis are coordinately regulated in human adipose tissue. J Nutr. 134: 1032-1038.

412. Wanke, I., M. Schwarz, and A. Buchmann. 2004. Insulin and dexamethasone inhibit TGF-beta-induced apoptosis of hepatoma cells upstream of the caspase activation cascade. Toxicology 204: 141-154.

413. Watanabe, S., Y. Horie, E. Kataoka, W. Sato, T. Dohmen, S. Ohshima, T. Goto, and A. Suzuki. 2007. Non-alcoholic steatohepatitis and hepatocellular carcinoma: lessons from hepatocyte-specific phosphatase and tensin homolog (PTEN)-deficient mice. J Gastroenterol. Hepatol. 22 Suppl 1: S96-S100.

414. Watanabe, T., M. Niioka, S. Hozawa, K. Kameyama, T. Hayashi, M. Arai, A. Ishikawa, K. Maruyama, and I. Okazaki. 2000. Gene expression of interstitial collagenase in both progressive and recovery phase of rat liver fibrosis induced by carbon tetrachloride. J Hepatol. 33: 224-235.

415. Weber, L. W., M. Boll, and A. Stampfl. 2003. Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. Crit Rev. Toxicol. 33: 105-136.

416. Wei, E., M. Alam, F. Sun, L. B. Agellon, D. E. Vance, and R. Lehner. 2007. Apolipoprotein B and triacylglycerol secretion in human triacylglycerol hydrolase transgenic mice. J. Lipid Res. 48: 2597-2606.

417. Wei, L., T. M. MacDonald, and B. R. Walker. 2004. Taking glucocorticoids by prescription is associated with subsequent cardiovascular disease. Annals of Internal Medicine 141: 764-770.

418. Weltman, M. D., G. C. Farrell, and C. Liddle. 1996. Increased hepatocyte CYP2E1 expression in a rat nutritional model of hepatic steatosis with inflammation. Gastroenterology 111: 1645-1653.

419. Westerbacka, J., H. Yki-Jarvinen, S. Vehkavaara, A.-M. Hakkinen, R. Andrew, D. J. Wake, J. R. Seckl, and B. R. Walker. 2003. Body fat distribution and cortisol metabolism in healthy men: enhanced 5β-reductase and lower cortisol/cortisone metabolite ratios in men with fatty liver. J. Clin. Endocrinol. Metab. 88: 4924-4931.

420. Wetterau, J. R., L. P. Aggerbeck, M. E. Bouma, C. Eisenberg, A. Munck, M. Hermier, J. Schmitz, G. Gay, D. J. Rader, and R. E. Gregg. 1992. Absence of

microsomal triglyceride transfer protein in individuals with abetalipoproteinemia. Science 258: 999-1001.

421. Williams, B. H. and C. D. Berdanier. 1982. Effects of diet composition and adrenalectomy on the lipogenic responses of rats to starvation-refeeding. J Nutr. 112: 534-541.

422. Wintersgill, H. P., P. Warburton, S. E. Bryson, S. G. Ball, and A. J. Balmforth. 1995. Glucocorticoids regulate the expression of angiotensin AT1 receptors, in the human hepatoma cell line, PLC-PRF-5. Eur. J Pharmacol. 288: 365-371.

423. Wolfe, R. R. 1992. Radioactive and stable isotope tracers in biomedicine. Principles and practice of kinetic analysis. New York: Wiley-Liss.

424. Wolfe, R. R. and E. J. Peters. 1987. Lipolytic response to glucose infusion in human subjects. Am J Physiol 252: E218-E223.

425. Wood, H. G., C. H. Werkman, A. Hemingway, and A. O. Nier. 1941. Heavy carbon as a tracer in heterotrophic carbon dioxide assimilation. J. Biol. Chem. 139: 365-376.

426. Xu, A., Y. Wang, H. Keshaw, L. Y. Xu, K. S. Lam, and G. J. Cooper. 2003. The fat-derived hormone adiponectin alleviates alcoholic and nonalcoholic fatty liver diseases in mice. J Clin Invest 112: 91-100.

427. Xu, J., G. Lee, H. Wang, J. M. Vierling, and J. J. Maher. 2004. Limited role for CXC chemokines in the pathogenesis of alpha-naphthylisothiocyanate-induced liver injury. Am J Physiol Gastrointest Liver Physiol 287: G734-G741.

428. Xu, Z., L. Chen, L. Leung, T. S. B. Yen, C. Lee, and J. Y. Chan. 2005. Liver-specific inactivation of the Nrf1 gene in adult mouse leads to nonalcoholic steatohepatitis and hepatic neoplasia. Proceedings of the National Academy of Sciences of the United States of America 102: 4120-4125.

429. Yamaguchi, K., L. Yang, S. McCall, J. Huang, X. X. Yu, S. K. Pandey, S. Bhanot, B. P. Monia, Y. X. Li, and A. M. Diehl. 2007. Inhibiting triglyceride synthesis improves hepatic steatosis but exacerbates liver damage and fibrosis in obese mice with nonalcoholic steatohepatitis. Hepatology 45: 1366-1374.

430. Yamauchi, T., J. Kamon, Y. Minokoshi, Y. Ito, H. Waki, S. Uchida, S. Yamashita, M. Noda, S. Kita, K. Ueki, K. Eto, Y. Akanuma, P. Froguel, F. Foufelle, P. Ferre, D. Carling, S. Kimura, R. Nagai, B. B. Kahn, and T. Kadowaki. 2002. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat. Med. 8: 1288-1295.

431. Yamazaki, Y., S. Kakizaki, N. Horiguchi, H. Takagi, M. Mori, and M. Negishi. 2005. Role of nuclear receptor CAR in carbon tetrachloride-induced hepatotoxicity. World J Gastroenterol. 11: 5966-5972.

432. Yasuhara, M., T. Ohama, N. Matsuki, H. Saito, J. Shiga, K. Inoue, K. Kurokawa, and T. Teramoto. 1991. Induction of fatty liver by fasting in suncus. J Lipid Res. 32: 887-891.

433. Yeager, M. P., P. M. Guyre, and A. U. Munck. 2004. Glucocorticoid regulation of the inflammatory response to injury. Acta Anaesthesiol. Scand. 48: 799-813.

434. Yen, C. L., S. J. Stone, S. Koliwad, C. Harris, and R. V. Farese, Jr. 2008. Thematic review series: glycerolipids. DGAT enzymes and triacylglycerol biosynthesis. J Lipid Res. 49: 2283-2301.

435. Yip, R. G. and H. M. Goodman. 1999. Growth hormone and dexamethasone stimulate lipolysis and activate adenylyl cyclase in rat adipocytes by selectively shifting Gi alpha2 to lower density membrane fractions. Endocrinology 140: 1219-1227.

436. Yoneda, M., K. Hotta, Y. Nozaki, H. Endo, T. Uchiyama, H. Mawatari, H. Iida, S. Kato, K. Fujita, H. Takahashi, H. Kirikoshi, N. Kobayashi, M. Inamori, Y. Abe, K. Kubota, S. Saito, S. Maeyama, K. Wada, and A. Nakajima. 2009. Association between angiotensin II type 1 receptor polymorphisms and the occurrence of nonalcoholic fatty liver disease. Liver Int. 29: 1078-1085.

437. Younossi, Z. M., A. M. Diehl, and J. P. Ong. 2002. Nonalcoholic fatty liver disease: an agenda for clinical research. Hepatology 35: 746-752.

438. Yu, J., E. S. Chu, A. Y. Hui, K. F. Cheung, H. L. Chan, W. K. Leung, G. C. Farrell, and J. J. Sung. 2007. Lipoprotein lipase activator ameliorates the severity of dietary steatohepatitis. Biochem. Biophys. Res. Commun. 356: 53-59.

439. Yu, J., E. Ip, A. Dela Pena, J. Y. Hou, J. Sesha, N. Pera, P. Hall, R. Kirsch, I. Leclercq, and G. C. Farrell. 2006. COX-2 induction in mice with experimental nutritional steatohepatitis: Role as pro-inflammatory mediator. Hepatology 43: 826-836.

440. Zammit, V. A. 1999. The malonyl-CoA-long-chain acyl-CoA axis in the maintenance of mammalian cell function. Biochem. J 343 Pt 3: 505-515.

441. Zeisel, S. H. and J. K. Blusztajn. 1994. Choline and human nutrition. Annu. Rev. Nutr. 14: 269-296.

442. Zelman, S. 1952. The liver in obesity. AMA. Arch. Intern. Med. 90: 141-156.

443. Zhang, T. Y., X. Ding, and R. A. Daynes. 2005. The expression of 11 beta-hydroxysteroid dehydrogenase type I by lymphocytes provides a novel means for intracrine regulation of glucocorticoid activities. J Immunol. 174: 879-889.

444. Zimmermann, R., J. G. Strauss, G. Haemmerle, G. Schoiswohl, R. Birner-Gruenberger, M. Riederer, A. Lass, G. Neuberger, F. Eisenhaber, A. Hermetter,

and R. Zechner. 2004. Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. Science 306: 1383-1386.

445. Zoppini, G., G. Targher, C. Venturi, C. Zamboni, and M. Muggeo. 2004. Relationship of nonalcoholic hepatic steatosis to overnight low-dose dexamethasone suppression test in obese individuals. Clin Endocrinol (Oxf) 61: 711-715.

446. Zou, Y., J. Li, C. Lu, J. Wang, J. Ge, Y. Huang, L. Zhang, and Y. Wang. 2006. High-fat emulsion-induced rat model of nonalcoholic steatohepatitis. Life Sci. 79: 1100-1107.